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# THE BOTANICAL GAZETTE

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## DEVELOPMENT OF CHERRY AND PEACH FRUITS AS AFFECTED BY DESTRUCTION OF THE EMBRYO<sup>1</sup>

H. B. TUKEY

(WITH SEVEN FIGURES)

### Introduction

That there is a relation between seed formation and fruitfulness is well known, to cite only the work of DETJEN (5) and KRAUS (8). Without fertilization, the peach, cherry, and plum fail to set fruit; and the apple and pear only rarely do so. Moreover it has been shown by many workers, including BRITAIN and EIDT (1), CRANDALL (2), KOBEL (7), and MURNEEK (14), that the total number of seeds which a fruit contains affects its performance and development. Apple fruits with a high seed content are less likely to absciss prematurely and are most likely to reach the size and shape typical of the variety.

There is, however, little information available upon the relation between fruit development and seed development, as distinct from the relationship between fertilization and set and development of fruit. TUKEY (17, 18) has shown that with the sweet cherry (*Prunus avium* L.) and peach (*P. persica* Stokes) there is a correlation between early ripening of the fruit and embryo abortion. Again it is common observation that insect attack upon developing fruits affects them differently. Fruits of the apple which are attacked severely by

<sup>1</sup> Journal Paper no. 134 of the New York State Agricultural Experiment Station.

aphids will cling to the tree tenaciously, whereas apples which are attacked by the codling moth drop or may ripen prematurely. Some of these insects attack the developing seed while others attack only the flesh.

All of these facts point to a direct relationship between fruit development and seed development. The work here reported was begun to determine the effect of destruction of embryos and seeds upon the development of growing fruits.

#### Development of embryo, seed, and pericarp of cherry and peach

It has been shown by CONNORS (3), HARROLD (6), LILLELAND (9), and TUKEY (18) that the fruits of the peach develop in three clearly defined stages, and by TUKEY (17, 19) that the fruits of the sweet and sour cherry behave similarly. In each there is a period of rapid development of the pericarp beginning about the time of full bloom (stage I), a period of retarded pericarp development during mid-season (stage II), and a period of rapid pericarp development just prior to fruit ripening, often referred to as the "final swell" (stage III).

The length of these periods varies with the variety, the species, and the season. The period of first rapid development of the pericarp (stage I) following full bloom is the most uniform. Its duration is nearly identical for the varieties in a given class of fruit (18, 19). That is, at Geneva, New York, stage I has a duration of seventeen days for the sour cherry (*Prunus cerasus*) regardless of the variation in time of fruit ripening of the different varieties. For the sweet cherry (*P. avium*) stage I continues for twenty-one days, and for the peach (*P. persica*) for forty-nine days (18). During this period the nucellus and integuments attain maximum size.

Stage II (period of retarded increase of the pericarp) is correlated directly with the season of fruit ripening. Early-ripening varieties have a short period of retarded development whereas late-ripening varieties have a longer period. In the case of the sour cherry, for example, the duration is five days for an early-ripening variety which attains full maturity forty-one days after full bloom; twelve days for a mid-season variety, ripening fifty-seven days after full bloom; and

twenty-eight days for a late variety, ripening sixty-six days after full bloom. At the beginning of this period the stony pericarp begins to harden.

Stage III (period of second rapid increase of pericarp) is variable and continues until final ripening.

The development of the embryo also progresses in three distinct stages, but not necessarily parallel with those of the pericarp (17, 18, 19). Following full bloom, while the pericarp and nucellus and integuments are in stage I, the embryo is in a state of arrested development which terminates abruptly in rapid increase, at just the time that stage II of development of the pericarp commences. The initiation of rapid increase of the embryo is correlated also with the initiation of hardening of the stony pericarp and with attainment of maximum size by the nucellus and integuments.

In late-ripening varieties of stone fruits, the rapid development of the embryo continues to maximum size during the period of retarded development of the pericarp (stage II). Seed formation proceeds to maturity, and late-ripening varieties of stone fruits are characterized by viable seed. On the other hand, in the case of early-ripening varieties, stage III of development of the pericarp begins before the embryo is full size, and such varieties are characterized by many abortive or non-viable embryos.

This situation raises several questions. Does the short duration of stage II in early-ripening varieties result in competition between the embryo and the pericarp and ultimate abortion of the embryo? Does the abortion of the embryo permit the initiation of stage III of development of the pericarp? If the embryo of a peach or cherry were destroyed at stage II of development of the pericarp, would the effect be to cause initiation of stage III and thus make a late-ripening variety ripen as an early variety? The material which follows attempts to answer these and related questions.

#### Materials and methods

Three varieties of sour cherry and six varieties of peach were used. The sour cherry varieties were Early Richmond, an early-ripening variety; Montmorency, a mid-season variety; and English Morello, a late-ripening variety. The peach varieties were Greensboro, Tri-

umph, Arp, and Lola, early-ripening varieties; Elberta, a late mid-season variety; and Chili, a late-ripening variety. The studies were conducted during the growing seasons of 1934 and 1935 in the varietal orchards of the New York State Agricultural Experiment Station at Geneva, New York.

Growth curves of these varieties have been established during several seasons at Geneva, New York (17, 18, 19). The growing season is such that most of the individual fruits on a given tree develop nearly identically. This is in part due to the fact that the blooming period is short, often only 36 to 48 hours, so that the fruits on a tree are fertilized and begin development at approximately the same time. This fact makes it possible to use many specimens for measurements and studies with small error from random sampling. In all this work several specimens have been used for each series of tests, involving between 500 and 600 sour cherry fruits and 300 and 400 peach fruits upon which individual growth measurements have been made periodically.

Growth of the pericarp has been recorded by measuring the length, suture diameter, and cheek diameter, and computing the mean. This figure has been shown to follow volume very closely (19). The growth in length of embryo, nucellus, and integuments has been recorded. Although figures are also available for both transverse diameters, they have not been used because length alone has seemed to give the desired information.

For establishing growth curves, measurements have been made on Monday, Wednesday, and Friday of each week from full bloom to fruit ripening, random samples being taken from the trees and critically measured and dissected in the laboratory (18, 19). In addition, individual fruits have been tagged on the trees and measured regularly throughout the season.

DESTRUCTION OF EMBRYO.—In order to destroy the seed and the embryos in developing fruits, both a needle and a small hand drill were used. The needle was satisfactory for reaching the seed through the distal end of fruits with the split pits characteristic of some varieties, as the Greensboro peach, and more or less common in other varieties depending upon the season (16). For most specimens, however, it was necessary to drill a tiny hole through the fleshy and stony



pericarp into the ovarian cavity, through which a needle could be jabbed or other treatment introduced.

A hand drill of the type shown in figure 1, with a drill having a diameter of 0.038 inch, was found satisfactory. From 200 to 300 drillings could be made in an hour by one individual.

To avoid bacterial or fungous contamination the drills were sterilized and the fruit washed with calcium hypochlorite (2 per cent chlorine) solution. In some instances a similar solution was introduced into the ovarian cavity, in others melted paraffin was used to cover and protect the wound, but the results showed no differences.



FIG. 1.—Hand drill (diameter 0.038 inch) used in destroying embryos

The drill method was finally adopted as standard technique, without sterilization and without wound protection. In most cases the fruit developed a wound callose.

Five different positions of drilling were used, only one position being used for any one fruit, namely, directed at (*A*) the embryo through the distal end of the fruit, (*B*) the hilum through the ventral suture, (*C*) the embryo through the cheek of the fruit, (*D*) the fleshy pericarp without injuring the stony pericarp and without entering the ovarian cavity, and (*E*) the stony pericarp but without entering the ovarian cavity (fig. 2).

Since the seed of the peach and cherry is anatropous with the embryo near the distal end of the fruit, drilling at position *A* strikes the embryo directly. Drilling at position *B* was seldom successful in severing the seed at the hilum, but accomplished the desired result by striking into the seed and the embryo. Drilling at position *C* was in most cases equally as effective as at positions *A* and *B*, by interfering with the seed and often striking the embryo. The most

satisfactory and most easily executed drilling to affect the embryo, however, was in position *A*. Drillings in positions *D* and *E* were made as checks to see what effect injury to the stony and fleshy pericarps incidental to destruction of the embryo might have upon fruit development.

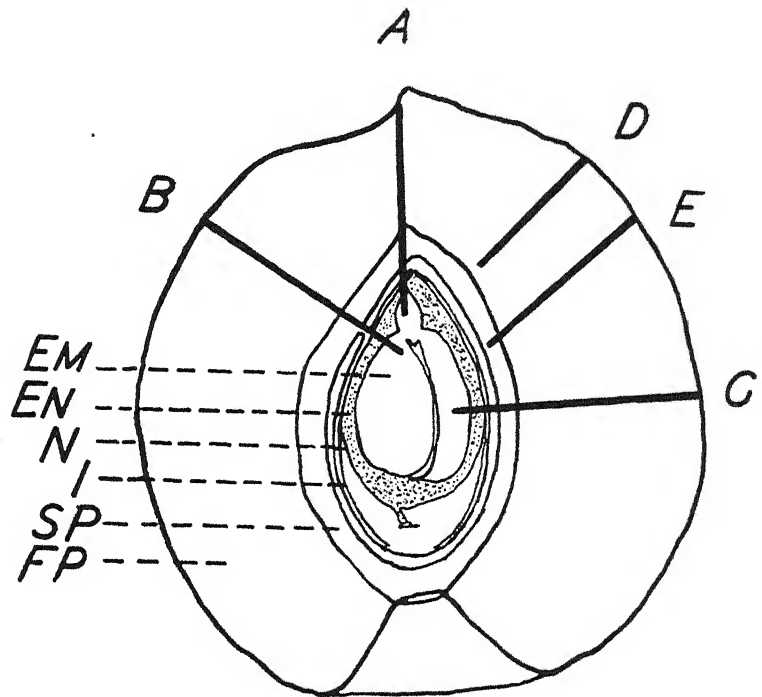


FIG. 2.—Diagram of developing peach fruit showing five positions used in drilling fruit and in destroying the partially developed embryos (*em*, embryo; *en*, endosperm; *n*, nucellus; *i*, integuments; *sp*, stony pericarp; *fp*, fleshy pericarp). Position *A*, through distal end of fruit into embryo; *B*, through ventral suture and hilum into embryo; *C*, through cheek of fruit into embryo; *D*, into fleshy pericarp without injuring seed; *E*, into stony pericarp without injuring seed.

## Results

### CHERRIES

Cherries were drilled June 5, 1934, in positions *A*, *B*, *C*, *D*, and *E* at the stages of development shown in table I, only one position of drilling being used for any one fruit.

Three days after drilling in positions *A*, *B*, and *C*, fruits of Early Richmond were turning yellow; six days after drilling they were coloring and softening, compared with the characteristic ripening date for the variety two days later. They did not, however, develop high cherry-red color. On the other hand, fruits of both Montmorency and English Morello were yellowing and shriveling three days after destruction of the embryo and were abscising three days later. Examination of the seeds three days after drilling showed them browning and shriveling, and six days after drilling both the embryo and the entire seeds were disintegrating.

TABLE I

STAGE OF DEVELOPMENT IN DRILLED CHERRY FRUITS OF PERICARP,  
NUCELLUS AND INTEGUMENTS, AND EMBRYO (JUNE 5, 1934)

VARIETY	MEAN DI-AMETER OF FRUIT (MM.)	CONDITION OF STONY PERICARP	LENGTH* OF NUCELLUS AND INTEGUMENTS (MM.)	LENGTH OF EMBRYO (MM.)	STAGE OF FRUIT DEVELOPMENT
Early Richmond.....	10.4	Hard	6.1	3.8 (abortive)	III
Montmorency.....	11.5	Hard	6.3	3.5	II
English Morello.....	12.1	Hard	7.2	3.3 (developing)	II

\* Maximum length for all varieties.

Fruit drilled in positions *D* and *E*, so as to injure the fleshy pericarp alone and the fleshy and stony pericarps together but without affecting the seed, grew normally and ripened at the usual time for the respective varieties. In other words, where destruction of the embryos of Early Richmond fruits just entering stage III resulted in earlier ripening, destruction of the embryos of Montmorency and English Morello on the same day, but in stage II, resulted in an abrupt check of fruit development and abscission.

#### PEACHES

Inasmuch as the destruction of the embryo of cherries indicated a difference in response between early-ripening and late-ripening varieties, this factor was more carefully considered in the tests with peaches. Peaches were drilled variously in positions *A*, *B*, *C*, *D*, and

*E* at the stages of development and on the dates shown in table II, only one position of drilling being used for any one fruit.

This plan included varieties ripening at different dates from early to very late and all treated similarly on the same date, and also included two varieties of long-growing season, treated similarly at dif-

TABLE II  
DATES AND STAGES OF DEVELOPMENT IN DRILLED PEACH FRUITS  
OF PERICARP, NUCELLUS AND INTEGUMENTS, AND EMBRYO

VARIETY	DATE	MEAN DI-AMETER OF FRUIT (MM.)	CONDITION OF STONY PERICARP	LENGTH* OF NUCELLUS AND INTEGUMENTS (MM.)	LENGTH OF EMBRYO (MM.)	STAGE OF FRUIT DEVELOPMENT
Triumph.....	July 26	49.2	Hard	16.5	9.5 (developing)	III
Greensboro...	July 26	56.3	Hard	21.0	12.5 (developing)	III
Arp.....	July 26	35.8	Hard	18.5	11.5 (developing)	III
Lola.....	July 26	47.6	Hard	.....	.....	III
Elberta (I)....	July 26	41.4	Hard	22.0	11.0 (developing)	II
Elberta (II)...	Aug. 14	45.2	Hard	22.0	12.5 (developing)	III
Elberta (III)...	Aug. 17	46.2	Hard	22.0	20.7 (full size)	III
Elberta (IV)...	Aug. 22	47.5	Hard	22.0	20.7 (full size)	III
Elberta (V)...	Aug. 29	49.6	Hard	22.0	20.7 (full size)	III
Chili (I).....	July 26	35.3	Hard	16.3	13.5 (developing)	II
Chili (II).....	Aug. 14	36.5	Hard	16.3	13.8 (developing)	II-III
Chili (III)....	Aug. 17	37.0	Hard	16.3	15.4 (full size)	II-III
Chili (IV).....	Sept. 12	46.5	Hard	16.3	15.4 (full size)	III

\* Full size for all varieties.

ferent dates. The early-ripening varieties were all in stage III when treated, while the Elberta and Chili varieties were in different points in stages II and III. The embryos were all in the period of rapid increase when the first drillings were done. In later drillings some of the embryos had approached full size and maturity. In all cases, even with the earliest drillings, nucellus and integuments had reached maximum size and the stony pericarp had begun to harden.

DESTRUCTION OF EMBRYO OF EARLY-RIPENING VARIETIES.—The performance of peach fruits following destruction of the embryo and wounding of the pericarp was similar to that of the sour cherry. In the case of such early-ripening varieties as Triumph, Greensboro, Arp, and Lola, destruction of the embryo resulted in earlier ripening of the fruits. In table III are given the data for the Triumph peach, which may be taken as representative of these four varieties, and with which the other varieties may be compared. Greensboro fruits with embryos destroyed July 26, ripened August 9, five days earlier

TABLE III

FRUIT DEVELOPMENT OF TRIUMPH PEACH AS AFFECTED BY KILLING  
THE EMBRYO AT DIFFERENT STAGES OF DEVELOPMENT  
(BLOOMED MAY 22; FRUIT RIPE, AUGUST 12, 1935)

TREATMENT	SIZE OF FRUIT (MEAN OF LENGTH, CHEEK DIAMETER, AND SUTURE DIAMETER) (MM.)		
	JULY 26	JULY 30	AUGUST 9
Embryo killed July 26			
Position A.....	49.2	53.22	66.45 (soft ripe)
Position C.....	49.2	53.10	66.35 (soft ripe)
Split-pit jabbed with needle.....	49.2	54.83	..... (soft, dropped)
Check (pericarp drilled in cheek three times, but embryo not injured)			
Position D.....	49.2	52.62	63.42 (hard ripe)
Check ("normal" fruit development)...	49.2	52.36	64.25 (hard ripe)

than untreated fruits; and Arp fruits with embryos destroyed, ripened August 14, eight days earlier than untreated fruits. Lola fruits with embryos destroyed August 17 ripened August 26, or three days earlier than untreated fruits.

Not only were the fruits of early varieties hastened in fruit ripening by destruction of the embryo, but also the growth rates were increased so that the fruits were larger on the same date than untreated fruits (fig. 3). Furthermore, fruits with embryos destroyed were larger in size *at maturity* than normal fruits *at maturity*, although the increase in size is not large (fig. 4). Fruits of Triumph with embryos destroyed attained at maturity a mean diameter 2 to 3 mm. greater than that of fruits not so treated, fruits of Greensboro

2 to 2.6 mm. greater, of Arp 5.74 mm. greater, and of Lola only 0.42 mm. greater.

**DESTRUCTION OF EMBRYO OF LATE-RIPENING VARIETIES.**—Destruction of the embryos of late-ripening varieties (Elberta and Chili) on the same day resulted in abrupt check in fruit development, shriveling, and abscission (figs. 5, 6, 7). The effect of embryo destruction was sharp, no measurable increase of fruit size occurring

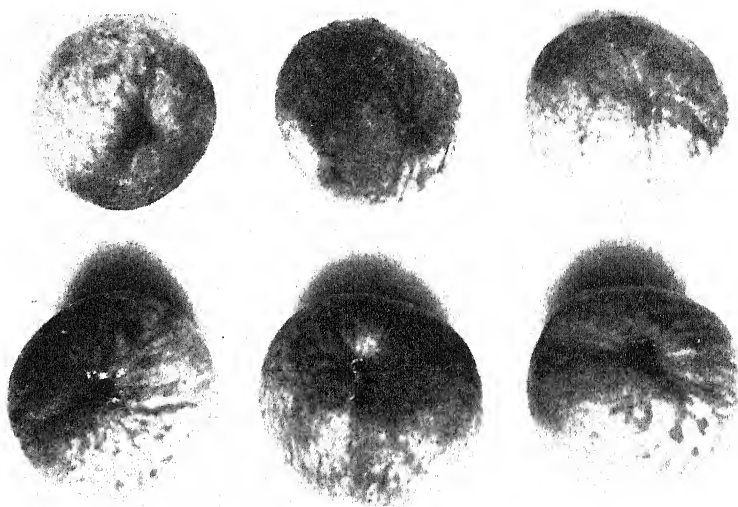


FIG. 3.—Fruit development of early-ripening variety (Triumph) following destruction of embryo during stage III of fruit development. Embryos destroyed by drilling July 26; fruits photographed August 10. Upper row not drilled; fruits developing typically and not ripe at this date. Lower row drilled; fruits larger, and ripe on same date. (Note drill holes in apex of lower row.)

thereafter (tables IV, V). It must be remembered, of course, that while the embryos of all varieties were in similar stages of development, yet the destruction of embryos of early-ripening varieties occurred in stage III whereas destruction of the embryos of late-ripening varieties on the same day occurred in stage II.

The results from destruction of the embryos of late-ripening varieties at points later along the curve of development differ still further in some respects from those of early-ripening varieties, whereas in other respects they agree. When embryos of Elberta were de-



stroyed August 14, 17, 22, and 29, the fruit was in stage III, and the result was to hasten maturity, just as in the case of the early-ripening varieties, whose embryos were destroyed in stage III. Furthermore, the nearer to the normal ripening date for the variety, the more abrupt was the increase in rate of growth from that of untreated

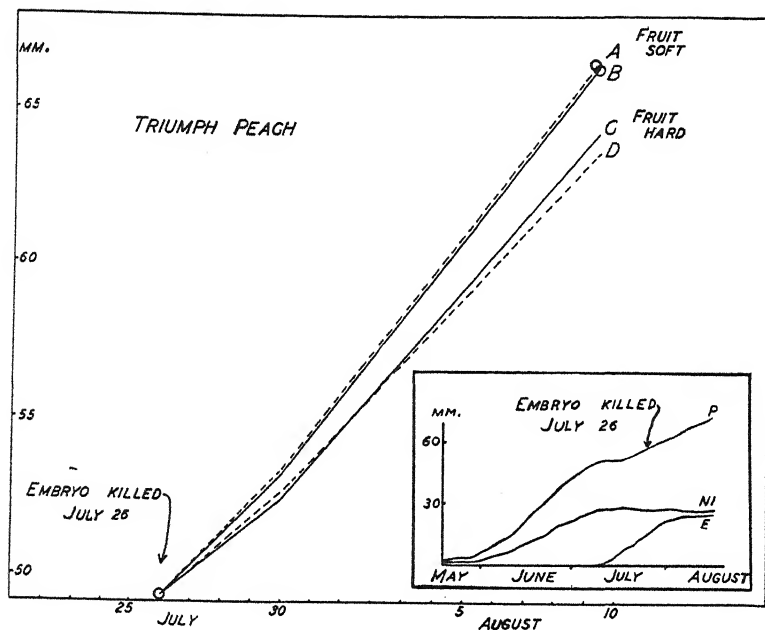


FIG. 4.—Growth of typical fruits of early-ripening variety of peach (Triumph), and of fruits with embryos destroyed by drilling July 26 at point in development indicated in insert (*p*, pericarp; *ni*, nucellus and integuments; *e*, embryo). Position *A*, embryo killed by drilling through distal end; *B*, embryo killed by drilling through cheek of fruit; *C*, pericarp wounded but embryo not injured; *D*, check.

fruit. Destruction at points early in stage III failed to increase the rate of growth although it did hasten ripening. Destruction at points later in stage III resulted not only in larger size at the time of ripening, as compared with untreated fruit on the same date, but also in what appears to be an actually larger size as compared with untreated fruit at maturity (fig. 6).

A further illustration of the more rapid increase in the rate of growth as the destruction of the embryo approaches fruit maturity



TABLE IV  
FRUIT DEVELOPMENT OF ELBERTA PEACH AS AFFECTED BY KILLING THE EMBRYO AT DIFFERENT STAGES OF DEVELOPMENT (FULL BLOOM MAY 20; FRUIT RIPE, SEPTEMBER 25, 1935)

TREATMENT	SIZE OF FRUIT (MEAN OF LENGTH, CHEEK DIAMETER, AND SUTURE DIAMETER) (MM.)								
	JULY 26	JULY 30	AUG. 9	AUG. 14	AUG. 17	AUG. 22	AUG. 29	SEPT. 12	SEPT. 25
I. Embryo killed July 26									
Position A.....	41.4	41.3 (shriveling)	30.3 (dropped)						
Position B.....	41.4	42.6 (shriveling)	..... (dropped)						
Position C.....	41.4	40.8 (shriveled)	..... (dropped)						
II. Embryo killed August 14									
Position A.....				45.2	46.0	47.9	53.1	62.2 (softening)	
III. Embryo killed August 17									
Position A.....					46.2	47.5	51.1	62.8 (coloring, softening)	
IV. Embryo killed August 22									
Position A.....						47.5	51.5	63.9 (softening)	
V. Embryo killed August 29									
Position A.....							49.6	62.7 (softening)	
Check ("normal" fruit development)	41.4	41.8	43.8	45.2	46.2	47.5	49.6	60.8	60.8 (ripe)
Check (pericarp drilled to pit but embryo not injured)									
Position E.....	41.4	41.0	44.3	44.6	45.7	47.0	50.0	60.9	60.9 (ripe)
Check (pericarp stung by insect but embryo not injured)									
Position D.....	41.4	41.4	43.8	45.5	45.6	47.4	50.0	61.1	61.1 (ripe)

TABLE V

FRUIT DEVELOPMENT OF CHILI PEACH AS AFFECTED BY KILLING THE EMBRYO AT DIFFERENT STAGES OF DEVELOPMENT (FULL BLOOM MAY 21; FRUIT RIPE, OCTOBER 4, 1935)

TREATMENT	SIZE OF FRUIT (MEAN OF LENGTH, CHEEK DIAMETER, AND SUTURE DIAMETER) (MM.)									
	JULY 26	JULY 3	AUG. 9	AUG. 14	AUG. 17	AUG. 22	AUG. 29	SEPT. 12	SEPT. 25	OCT. 4
I. Embryo killed July 26 Position A.....	35.3	35.6 (shriveled)	29.8 (dropped)							
II. Embryo killed August 14 Position A.....				36.5	36.7	37.7	40.0	46.4 (dropped)		
Check ("normal" fruit development on same branch)....										
III. Embryo killed August 17 Position A.....				36.5	36.8	37.3	38.3	44.0	45.4	45.4
IV. Embryo killed September 12 Position A.....					37.0	37.8	40.2	44.0 (dropped)		
Check ("normal" fruit development on same branch)....								46.5	51.3 (ripe, dropped)	
Check ("normal" fruit development)....	35.3	35.3	35.6	36.5	37.0	37.6	39.8	46.5	49.3	49.3 (soft- ening)

is seen from table IV of the Elberta peach. For example, fruits with embryos destroyed on August 14, 17, 22, and 29 all reached the stage of "soft ripe" on the same date, September 12. The fruits drilled on August 14, however, reached this stage by an increase between August 29 and September 12 of only 9.08 mm. in mean diameter, whereas the fruits drilled August 17 increased 11.67 between these dates, those drilled August 22 increased 12.45, and those drilled August 29 increased 13.15 mm.

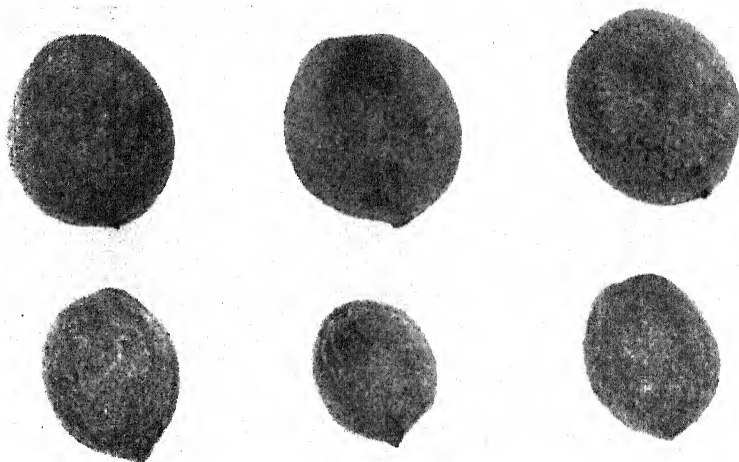


FIG. 5.—Fruit development stopped by destruction of embryo of late-ripening variety (Chili) during stage II of fruit development. Fruits drilled July 26; photographed August 10. Upper row not drilled, fruits developing typically. Lower row drilled, fruits shriveled and abscised on same date.

The results with the other late-ripening variety, Chili, also differ from those of the other varieties in some respects and agree in others (table V). They agree with Elberta in that the destruction of the embryo in stage II resulted in immediate check in fruit development, with subsequent shriveling and abscission (fig. 5). They agree with all varieties in that the destruction of the embryo late in stage III resulted in an increase in the growth rate and a hastening of ripening (fig. 7).

Most significant, however, are the points of embryo destruction at stages midway between early and late development. It will be re-

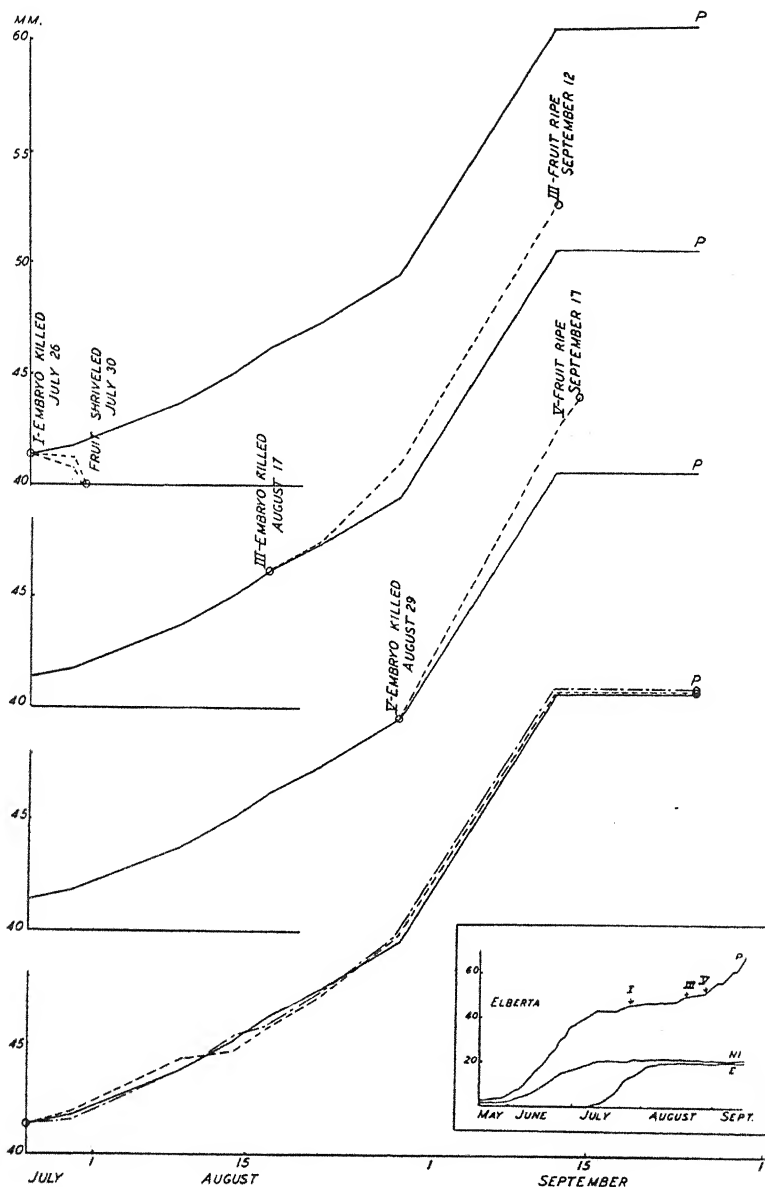


FIG. 6.—Growth of typical fruits of late mid-season variety of peach (Elberta) and of fruits with embryos destroyed by drilling at points in development indicated in insert (*p*, pericarp; *ni*, nucellus and integuments; *e*, embryo).

Solid lines=normal growth curve.

Dotted lines I, III, V=development following destruction of embryos at dates indicated.

Dotted lines in bottom curve=pericarp wounded by insect.

Broken line in bottom curve=pericarp wounded by drilling.

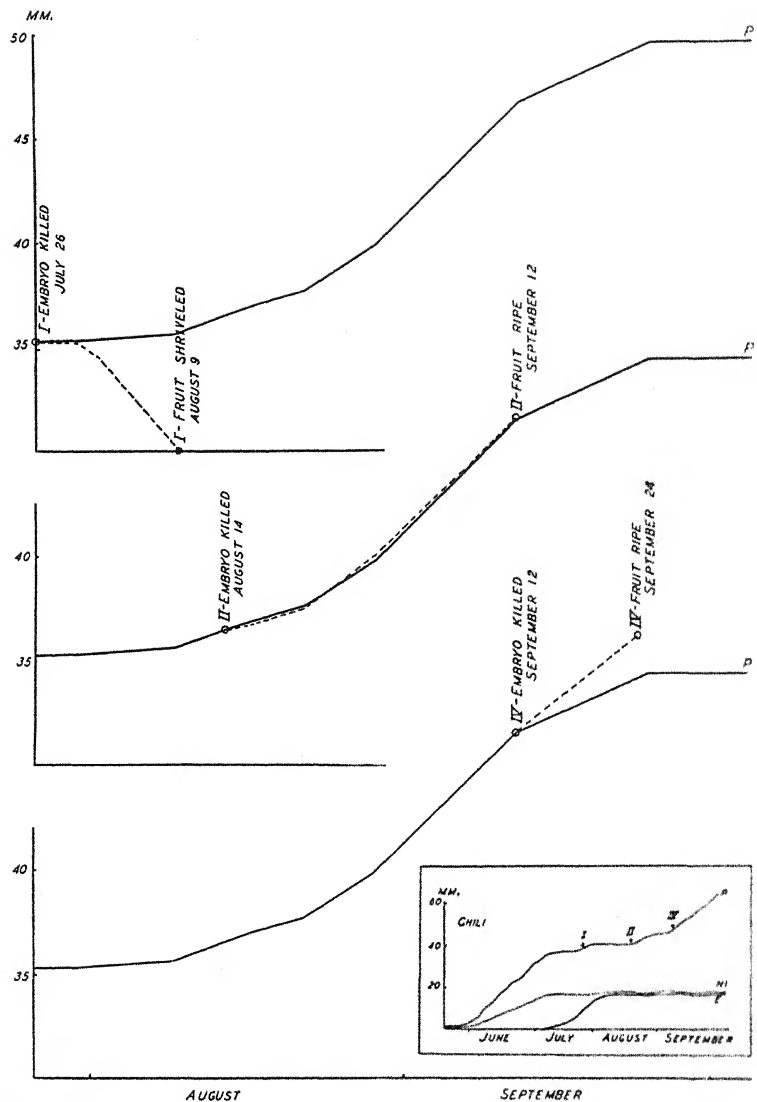


FIG. 7.—Growth of typical fruits of late-ripening variety of peach (Chili) and of fruits with embryos destroyed by drilling at points in development indicated in insert (p, pericarp; ni, nucellus and integuments; e, embryo).

Solid lines=normal growth curve.

Dotted lines I, II, IV=development following destruction of embryos at dates indicated.

membered that stage II of Chili is of relatively long duration. Apparently the destruction of the embryos on August 14 and 17 was at a point in development of the pericarp in transition between stages II and III, because while fruits with destroyed embryos increased in size after treatment, yet such fruits failed to reach full size for the variety and dropped prematurely. Accordingly they were unlike fruits whose embryos had been destroyed at earlier dates, in that they followed the growth curve of untreated fruits for a period whereas the others were checked abruptly in development and later shriveled and abscised. Also they were unlike fruits whose embryos were destroyed at dates near to maturity of untreated fruits, in that they failed to increase at a rate faster than the untreated fruits although they ripened earlier. Likewise they did not reach full size for the variety. They more nearly approached the behavior of Early Richmond fruits whose embryos were destroyed early in stage III.

WOUNDING OF THE PERICARP.—Quite in contrast to the results from destruction of the embryo were the results from wounding the fleshy pericarp alone and of the stony and fleshy pericarps together but without affecting the seed. Neither the growth rate of the fruit nor the season of fruit ripening was altered from that which is typical of the variety. In fact, in several instances observations were made of the successful and apparently normal development of fruits which were wounded in the flesh by birds and insects. One such instance was carefully measured and followed to maturity, as shown in table IV. The curve of growth and season of ripening were not affected.

In several instances treated and untreated fruits were adjacent on the same branch (table IV). In all such instances the fruits behaved in a manner characteristic of the type of treatment, thus eliminating any possibility of differences in nutritional effect or effect due to position on the tree.

#### Discussion of data

In most cases, fruits of varieties with different ripening dates did not respond to destruction of the embryo by ripening on the same date. Instead the ripening processes were hastened along the growth curve characteristic of the variety. For example, typical untreated fruits of Greensboro ripened August 14, and those of Arp, August 22.



Destruction of embryos of both varieties on July 26 did not result in fruits ripening on the same day; instead those of Greensboro ripened August 9, and those of Arp, August 12. Again fruits of Lola and of Elberta with embryos destroyed on August 17 ripened on August 29 and September 12 respectively, and fruits of Chili similarly treated failed to reach full size by September 12.

Yet the embryos of Chili were apparently as fully developed morphologically as those of Elberta on the same date, so that it cannot be said that destruction of the embryo is the sole controlling factor in development of the pericarp. Apparently there is a relationship between fruit development and embryo development which is characteristic of the variety, and which is altered by destroying the embryo, but which alone does not control the process of fruit development.

An additional consideration suggested by these data is the effect of such factors as frost and insect attack upon the development of the embryo, affecting in turn the development of the fruit. It might be expected that those agencies which destroy the embryo in early stages of development might cause the fruit to drop, whereas those which affect the fruit without destroying the embryo might not. Again, agencies which destroy the embryo in late stages of development might be expected to increase the growth rate and hasten the ripening of the fruit. General observations confirm these suggestions. Spring frosts which destroy the embryo frequently result in abscission of the fruit. Injury by frost to the pericarp or accessory fruit parts without injuring the embryo, however, does not result in premature abscission, although fruits so injured may display at maturity cracks, russet markings, and other evidences of early season injury. The attacks of the larvae of the codling moth (*Carpocapsa pomonella* L.), which in early season destroy the developing embryo, result in abscission of the fruits. Attacks from the larvae in late season, when the fruits are nearing maturity, hasten ripening of the fruit.

Observations also suggest a relation between pit splitting in peaches and the development of the embryo and the fruit. Among those studying this situation, DAVIS (4) has found a close relationship between the size or growth rate of fruits and the occurrence of



split-pits, in which large fruit sizes are associated with pit splitting. Moreover, he cites MIKI (12) as reporting that the percentage of split-pit is higher in the earlier ripening fruits within the same variety. DAVIS concludes, "These facts would seem to offer additional weight to the suggestion that the occurrence of split-pit is very closely associated with those conditions which cause an increased rate of growth of the fruit."

Subsequently RAGLAND (16), in a morphological study of split-pit, has reported that "embryos of split-pit fruits are aborted in a very high percentage of cases; nevertheless, the flesh develops to maturity as in normal fruits." He has found a group of bundles which he calls funicular bundles, supplying the ovules and anastomosing with the vascular cylinder at the base of the carpel, as distinct from the dorsal bundle lying along but never within the pit and the two ventral bundles lying in the deep grooves of the pit along the ventral suture.

Lignification of the pit does not proceed uniformly but begins along the ventral suture. Since there is little actual union between carpel edges, this region is subject to breakage from stresses set up either by the developing pericarp or by contraction within the pit itself (13, 16). The location of the funicular bundles so near the line of cleavage along the ventral suture renders them especially susceptible to rupturing when the split occurs.

These facts are suggestive of a situation in which splitting of the pit results in checking of the embryo as in early-ripening varieties, or as when the embryo is destroyed by external agencies, as drilling, resulting in turn in a more rapid growth rate of the fruit and earlier maturity.

Other factors, however, may play a part in the results brought about by destruction of the embryo. Among these are the morphological nature of the fruit. In the case of a simple fruit consisting of a single carpel, as in the peach, cherry, and plum, the effect from destruction of the embryo is less easily masked than in the case of multiple or accessory fruits consisting of several carpels and associated stem and receptacle, as the apple and the pear. Destruction of the single developing embryo in a peach might be expected to result in abscission of the fruit, whereas destruction of several or all of the

embryos of an apple might not result in abscission of the fruit, although possibly influencing the development of accessory fruit parts.

Furthermore, the genetic quality of a variety may alter the situation, as suggested by KRAUS (8). Thus, the McIntosh apple is self-sterile and self-unfruitful and seldom develops fruits without seed. On the other hand, the Baldwin apple may develop fruits containing only a few seeds, or may even develop fruit parthenocarpically. A further expression of this situation in these varieties is seen in the development of individual carpels following the failure of the embryo. In the case of the McIntosh apple, carpels which do not contain at least one seed are likely to be poorly developed, resulting in angular, lopsided, and otherwise misshapen fruits; while with the Baldwin apple, carpels which contain no developing seed may make growth and development resulting in well shaped fruits typical of the variety. Nutritional factors also play an important part, as when the number of apples which form following fertilization is greater than the tree can develop to maturity. In such case the fruits which drop in the various waves of early-season abscission are likely to be those with low seed count, whereas when the number of apples which form is small in proportion to the total number which the tree is able to develop, many fruits of low seed count may adhere and develop to maturity.

Yet factors which affect the fruit and the embryo equally without injury, such as temperatures, do not disturb the relation between embryo and fruit development. LILLELAND (10) in a study of temperature upon fruit development has found that when individual apricot fruits are inclosed in heating chambers, stages I and II of fruit development are accelerated in comparison with adjacent fruits untreated on the same branch. Likewise the stages of development of the embryo are accelerated and in direct relation to the increase in growth of the fruit, so that although treated fruits ripen earlier than untreated fruits, the embryos of treated fruits also develop earlier and are at the stage of development characteristic of the variety at maturity.

#### General discussion and conclusions

The results presented in this paper have been based largely on morphological observations. Certain physiological features remain.

Among these is the chemistry of "stage of development" as indicated by LOTT (11). That is, although morphologically embryos of Elberta and Chili may seem to be at identical stages of development on a given date, it does not necessarily follow that there may not be some more rapid accumulation or release of materials in one than in the other, or some other biological factor present in one and not in the other, which may be the controlling factor.

MURNEEK (14), in discussing the effects of seed development on the subsequent behavior of flowers, uses the results obtained with *Cleome spinosa* to show that the developing seeds of a plant may be responsible for the reduction in growth of the female reproductive organs, and in this instance bring about sterility. Removal of the fertilized pistils or young seed pods at the proper time resulted in all flowers developing normally and in being fertile. In elaborating upon growth and development as influenced by fruit and seed formation he says, "The question naturally arises as to what may be the means by which the developing embryos are able to draw to themselves and the adjoining tissues most of the available food supply. To accomplish such results a definite and powerful mechanism certainly must be at the disposal of the reproductive organs. At present we are entirely ignorant as to its nature beyond the mere conjecture that it may be either of a general physiological or a specific enzymatic or hormonal character."

The data presented in this paper, while not adding information that might help to answer this question, do support the theory propounded by MURNEEK and others and carry the principle to the point where embryo development is shown not only to influence plant development in general but also markedly to influence fruit development.

In answer to the question raised by TUKEY (17, 18) as to whether embryo abortion is responsible for the accelerated growth of pericarp in early-ripening varieties of cherry and peach, or whether it is the rapid growth of the pericarp which brings about embryo abortion, these results indicate that the embryo is the controlling factor.

Do they mean, however, that the pericarp does not develop in its final rapid growth just preceding maturity until the embryo "releases" it, or until some balance is attained between pericarp and seed parts, or until some substance is developed within the seed

parts which favor development of the pericarp? And do they mean that in the case of early-ripening varieties the release comes earlier so that balance is attained earlier, or a growth promoting substance is developed earlier than in the case of late-ripening varieties? It would seem that there are at least two factors in operation in this situation, the stage of development at which embryo destruction occurs and the characteristics of the variety.

### Summary

1. Development of the fruit of the cherry and peach is in three clearly marked stages: stage I, rapid increase of the pericarp from about the time of fertilization to mid-season; stage II, retarded increase during mid-season; and stage III, second rapid increase to fruit maturity.

2. Embryos of three varieties of sour cherry (*Prunus cerasus*) and six varieties of peach (*P. persica*) were destroyed at various stages of development by jabbing through split pits with a needle and by drilling through the pericarp. Fruits which were wounded in the fleshy pericarp and stony pericarp but without injury to the embryo, and fruits which were attacked by insects, were also followed in development.

3. Destruction of the embryo early in stage II of fruit development resulted in abrupt check in fruit development, shriveling, and abscission.

4. Destruction of the embryo in the transition between stages II and III of development of the pericarp resulted in growth of the fruit at a rate similar to that of untreated fruit for a limited period, but ended with earlier ripening and failure to reach full size.

5. Destruction of the embryo in stage III of development of the pericarp resulted in increased growth rate and earlier ripening, and occasionally increased size over that attained by untreated fruits at maturity.

6. Wounding of the fleshy and stony pericarps, without injuring the seed, did not alter the growth rate of the fruit.

7. The nearer the ripening date typical of the variety at which the embryo was destroyed, the greater was the increase in growth rate of the pericarp.



8. Two factors seem involved in the relation between embryo and pericarp: (a) the stage of development of the fruit and of the embryo at which the embryo is destroyed, and (b) the genetic makeup of the variety controlling this relation.

9. Development of the embryo is shown to bear a definite relation to the development of the fruit quite distinct from the effect of fruit and seed formation upon the entire plant.

10. In early-ripening varieties of cherries and peaches it would seem that abortion of the embryo is the factor which affects pericarp development rather than the reverse.

11. Frost, insect attack, and pit splitting of peaches are discussed in relation to their effect upon the development of the embryo and of the fruit. The morphological nature of fruits is considered in this regard, together with nutritional factors.

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## CONTROL OF ATMOSPHERIC HUMIDITY IN CULTURE STUDIES

HENRY HOPP

(WITH ELEVEN FIGURES)

The objectives of this paper are threefold: (a) to present a summary of the methods which have been used to control relative humidity; (b) to report several new procedures for their practical application; and (c) to consider their relative value in culture studies. The instruments and new procedures reported in this paper have been developed in studies concerned with the culture of wood-destroying fungi.

Control of the atmospheric humidity within culture chambers can be secured by chemical and physical means.

### Chemical control of humidity

Chemical control of the humidity is attained through the evaporation of water from a solution, the vapor pressure of which is regulated by the presence of dissolved chemicals. Various relative humidities may be secured quantitatively through the use of the same solute in different concentrations, or qualitatively by using different kinds of solutes in saturated solutions.

### QUANTITATIVE CHEMICAL CONTROL

Any soluble chemical which causes an appreciable reduction in aqueous vapor pressure may be used to obtain quantitative chemical control of the humidity (13). Sulphuric acid (5, 9, 15, 18, 20, 22) is most generally used. Sodium chloride has also been employed to secure percentages of relative humidity between 75 and 100 (4). The relation between relative humidity and concentration of sulphuric acid is shown in figure 1. The relative humidity secured at a definite concentration of acid is approximately constant (20) through the usual temperature range of culture incubation (15° to 30° C.), although it is best to maintain a constant temperature. For experi-



ments of short duration, the method can be employed very conveniently in a set-up using petri dishes (fig. 2). The dishes used are of two sizes, the smaller one (which is uncovered) being placed within the larger. The substratum is contained in the inner dish and the solution is poured into the outer one. The presence of sul-

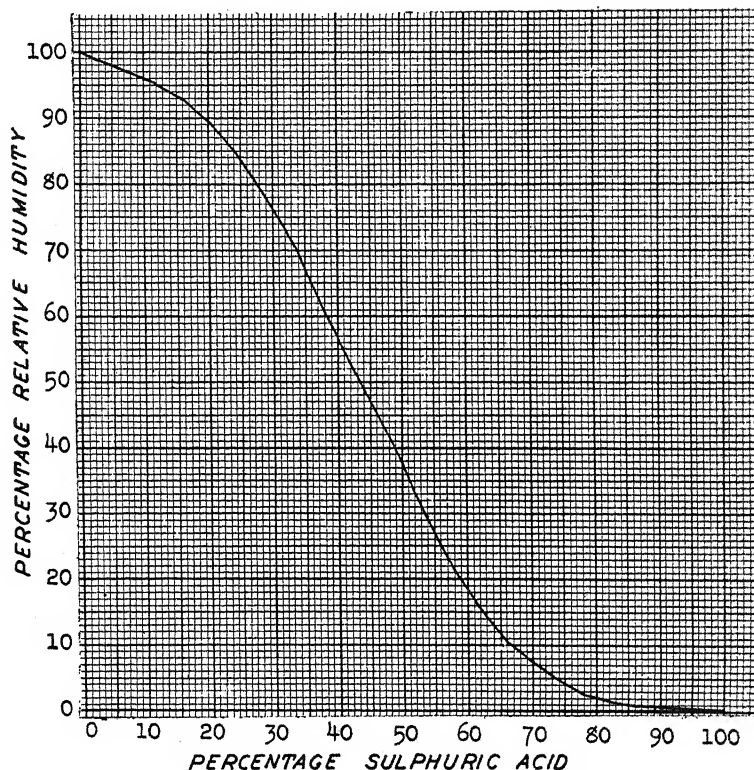


FIG. 1.—Relative humidity of atmosphere obtained above sulphuric acid solutions of various concentrations. Data from STEVENS (15).

phuric acid, the vapor pressure of which is practically nil, has no apparent effect on fungi growing in the inner dish.

The quantitative method of control, although simple and inexpensive, is of limited application. The relative humidity can be regulated effectively only in chambers of small volume, since diffusion from the surface of the solution is slow. If the water exchange between the air and the solution is considerable, the resulting change

in concentration of the solution will affect the relative humidity. A digression of 1 per cent from the original concentration of a sulphuric acid solution causes a variation in relative humidity of 1 to 2 per cent at ordinary temperatures. In order to prevent a significant change in concentration of the solution, therefore, the humidity chambers must be closed to the outside atmosphere. This is a handicap when living organisms are the subject of experimentation, since under certain conditions their respiratory processes cause a considerable change in the chemical composition of the air. The extent of this change was determined by measuring the oxygen consumption of various fungi. It was found, for example, that the

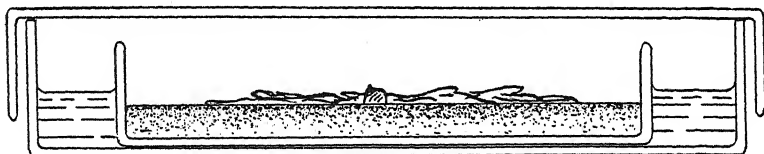


FIG. 2.—Petri dish set-up for culturing organisms under conditions of controlled atmospheric humidity.

oxygen content of the air within a Novy jar of 2.5 liters capacity which contained two petri-dish cultures of *Polyporus versicolor* (L.) Fries was decreased by 5 per cent in 24 hours; and after 5 days the oxygen content was only 0.56 per cent.

Control is ineffective also when large quantities of hygroscopic or hydrated material are present in the chamber which contains the solution. A hygroscopic material, such as dry wood blocks, if placed within chambers operated at a high relative humidity, would absorb considerable atmospheric water. As a result the concentration of the sulphuric acid solution would be significantly increased. Hydrated materials, such as agar media, if placed in chambers operated at a low relative humidity, would cause considerable dilution of the sulphuric acid solution. When small quantities of agar medium are used, however, the dilution of the solution is so slight that the relative humidity is not considerably changed.

#### QUALITATIVE CHEMICAL CONTROL

Saturated solutions of various chemical substances (table I) may be used to control the humidity of the atmosphere (5, 6, 1, 7, 19).

The mechanics of the method are relatively simple. A saturated solution of the substance is made and an excess of the solute is then

TABLE I  
APPROXIMATE RELATIVE HUMIDITY OF  
ATMOSPHERE AT ROOM TEMPERATURE  
OBTAINED ABOVE SATURATED SOLUTIONS OF VARIOUS SUBSTANCES. DATA  
FROM SPENCER (13)

SUBSTANCE	PERCENTAGE RELATIVE HUMIDITY
$\text{CaSO}_4 \cdot 5\text{H}_2\text{O}$ .....	98
$\text{K}_2\text{SO}_4$ .....	98
$\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$ .....	95
$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ .....	95
$\text{KNO}_3$ .....	94
$\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$ .....	93
$\text{K}_2\text{HPO}_4$ .....	92
$\text{Na}_2\text{C}_4\text{H}_4\text{O}_6 \cdot 2\text{H}_2\text{O}$ .....	92
$\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$ .....	88
$\text{K}_2\text{CrO}_4$ .....	88
$\text{Na}_2\text{CO}_3 \cdot 10\text{H}_2\text{O}$ .....	88
$\text{KCl}$ .....	87
$\text{KHSO}_4$ .....	86
$\text{KBr}$ .....	84
$\text{Na}_2\text{SO}_4$ .....	82
$\text{NH}_4\text{Cl}$ .....	79
$\text{H}_2\text{C}_2\text{O}_4 \cdot 2\text{H}_2\text{O}$ .....	76
$\text{NaCl}$ .....	76
$\text{NaNO}_3$ .....	76
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ .....	67
$\text{NaNO}_2$ .....	66
$\text{NH}_4\text{NO}_3$ .....	64
$\text{NaBr} \cdot 2\text{H}_2\text{O}$ .....	58
$\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ .....	53
$\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ .....	52
$\text{Na}_2\text{Cr}_2\text{O}_7 \cdot 2\text{H}_2\text{O}$ .....	52
$\text{NaHSO}_4 \cdot \text{H}_2\text{O}$ .....	52
$\text{KNO}_2$ .....	45
$\text{K}_2\text{CO}_3 \cdot 2\text{H}_2\text{O}$ .....	43
$\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ .....	42
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ .....	33
$\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ .....	32
$\text{KC}_2\text{H}_3\text{O}_2$ .....	20
$\text{LiCl} \cdot \text{H}_2\text{O}$ .....	15
$\text{H}_3\text{PO}_4 \cdot \frac{1}{2}\text{H}_2\text{O}$ .....	9

added. In this way permanent saturation is obtained, since absorption of atmospheric water into the solution causes dissolution of a portion of the excess solute.

Owing to the slow rate of diffusion from the surface of the solution, effective regulation of the atmospheric humidity can be attained only in chambers of small volume. This disadvantage can be overcome either by using fans in the humidity chamber or by humidifying a current of air, which is then passed through the chamber. Continuous replenishment of the air is especially desirable when rapidly respiring material is the subject of experimentation (6). A method to procure continuous renewal of the air within the chamber is shown in figure 3. The air is bubbled through a saturated

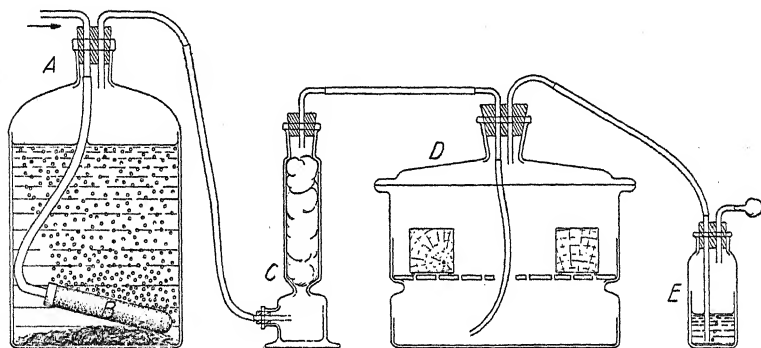


FIG. 3.—Qualitative chemical control of humidity in fresh-air chambers under sterile conditions: *a*, humidifying jar; *b*, diatomaceous filter; *c*, cotton filter; *d*, humidity chamber containing experimental material; *e*, trap-jar.

salt solution, which is contained within a humidifying jar. It is then filtered in a tower packed with absorbent cotton in order to remove suspended droplets of the solution. The conditioned air passes into the humidity chamber and is discharged through a trap-jar containing some of the salt solution. Thorough humidification of the air is assured by dividing it into small bubbles within the humidifying jar. A diatomaceous filter is satisfactory for the purpose. An aquarium aerator consisting of a 1-inch cube block of porous carborundum, into which a piece of glass tubing is cemented, or simply a block of porous-veined wood sealed into the end of a rubber tube, can also be used. The humidity chamber is freed of organisms by sterilization with acetic acid, which may be evaporated by operating the apparatus for several days before the start of the experiment.

All contaminants are caught in the salt solution and in the cotton filter.

The apparatus requires little attention once it is adjusted. The velocity of the air stream can be regulated by either a pet-cock or a pressure regulator on a compressed air line. The rate of bubbling in the trap-jar indicates the air velocity. The pressure required in the air line depends on the kind of porous block employed in the humidifying jar. A diatomaceous filter requires a minimum pressure of about 12 pounds per square inch. Five pounds' pressure is needed to force the air through an aquarium aerator. When the humidity of the conditioned air exceeds that of the air supplied by the pressure line, the porous block becomes clogged. This is due to the absorption of water from the saturated solution and the consequent crystallization of solute. To avoid this situation, the air should be humidified before it passes through the porous block. A simple way to saturate the air is to bubble it through a small jar of water.

The writer has found this apparatus entirely dependable in tests conducted over a period of several years. With this set-up the air is not only humidified but is also sterilized. The method is well suited for the control of humidity in studies involving gas exchange (19).

In determining the substance to be used in attaining a desired relative humidity, considerable selection is often possible. Three factors should be considered in making this choice: (1) the solubility of the solutes, (2) the effect of temperature on the change in their solubility, and (3) the influence of temperature changes on the aqueous vapor pressure of their saturated solutions. The effects of these factors are illustrated in the case of the choice between  $\text{NaNO}_3$  and  $\text{NaCl}$ , either of which can be used to obtain a relative humidity of 76 per cent at  $23^\circ \text{C}$ . (fig. 4). Owing to the difference in solubility of these salts, it is necessary to use about twice as much  $\text{NaNO}_3$  as  $\text{NaCl}$  in order to prepare saturated solutions. On the basis of expense and convenience alone, the latter salt would be preferred. The effect of temperature variation on the solubility of the salt is another important factor. If a slight fall in temperature causes a relatively great decrease in solubility, a considerable amount of the salt will be precipitated out of the solution. The physical effect of

this result is important since the solute is precipitated not only on the bottom of the humidifying jar but also on the porous block, which then becomes clogged. On the other hand, if the temperature rises, a considerable amount of the excess salt lying at the bottom of the humidifying jar will be dissolved. During the period of time

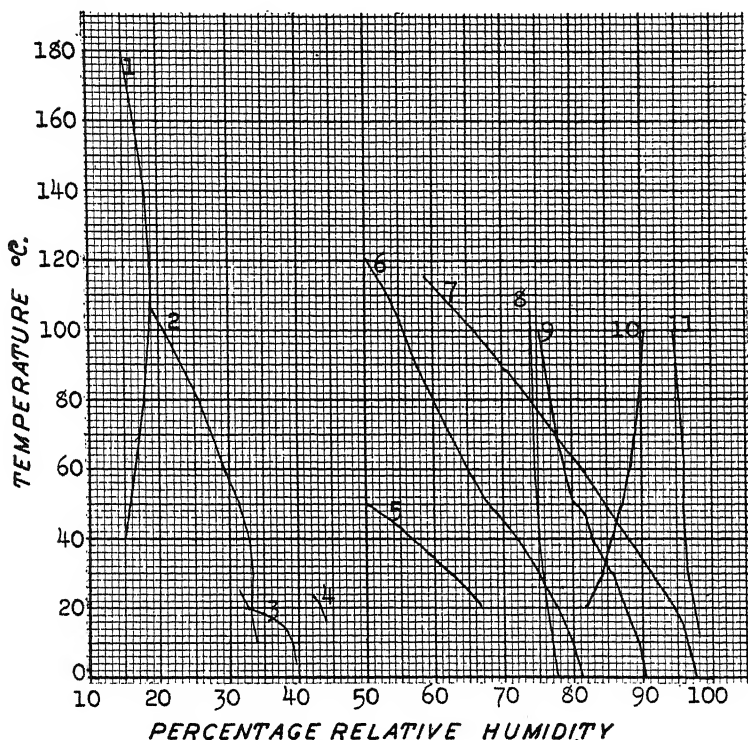


FIG. 4.—Effect of temperature on relative humidity obtained with saturated solutions of various substances: (1)  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , (2)  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , (3)  $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ , (4)  $\text{K}_2\text{CO}_3 \cdot 2\text{H}_2\text{O}$ , (5)  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , (6)  $\text{NaNO}_3$ , (7)  $\text{KNO}_3$ , (8)  $\text{NaCl}$ , (9)  $\text{KCl}$ , (10)  $\text{Na}_2\text{SO}_4$ , (11)  $\text{K}_2\text{SO}_4$ . From SPENCER (13).

required to accomplish the change, the solution is unsaturated and the aqueous vapor pressure is increased. This results in a temporary increase of the relative humidity. The average change of solubility in 100 gm. of water for each degree change in temperature between 0 and 100 is 0.04 gm. for  $\text{NaCl}$  and 1.07 gm. for  $\text{NaNO}_3$ . Because of

the smaller change in solubility, therefore, NaCl would be the salt preferred. Another advantage in using NaCl is that the aqueous vapor pressure in a saturated solution of this salt is affected much less by changes in temperature than is that in a  $\text{NaNO}_3$  solution. As a result of the variation in aqueous vapor pressure, the relative humidity above a NaCl solution changes from 77 to 75 per cent between  $10^\circ$  and  $40^\circ$  C., which are the usual extreme temperatures of incubation. In the case of  $\text{NaNO}_3$ , the relative humidity obtained is 80 per cent at  $10^\circ$  and changes to 72 per cent at  $40^\circ$  C. The vapor pressure of the salts is so small that this factor is of no importance, and need not be considered in evaluating the method.

#### Physical control of humidity

Physical control is achieved by electrical or mechanical regulation of the rate of humidification. If vaporization of the water within the humidifier proceeds continuously, the air stream will be saturated, whereas intermittent vaporization results in the air being saturated only part of the time.

#### CONTINUOUS VAPORIZATION

The method of continuous vaporization is easily applied if a saturated atmosphere is desired, but it is rather difficult to use when relative humidities of less than 100 per cent are required. In closed chambers the air can readily be saturated by evaporation from a free water surface or from wet strips of cloth (22). This procedure, although simple, has the disadvantage that the water from the evaporating surface will condense on the experimental material and on the sides of the container unless the temperature is held absolutely constant. A simple variation which permits the use of fresh-air chambers is to blow the air through a humidifier containing water (12). When the velocity of the air stream is small, a glass tower containing wet cloth can be used for humidification (fig. 5). To saturate large volumes of air, a water spray is often employed (2, 7, 8, 14).

By this method two procedures are available to secure relative humidities less than saturation. One way is to saturate the air at a low temperature and then reheat it. The relative humidity ob-



tained will depend on the initial and end temperatures (fig. 10). This method is applicable in commercial work but the large amount of complicated control equipment required generally makes it impractical for use in culture experiments. The second way is to mix saturated air in definite proportions with dry air or with air of a fixed relative humidity. The air can be dried by passing it through

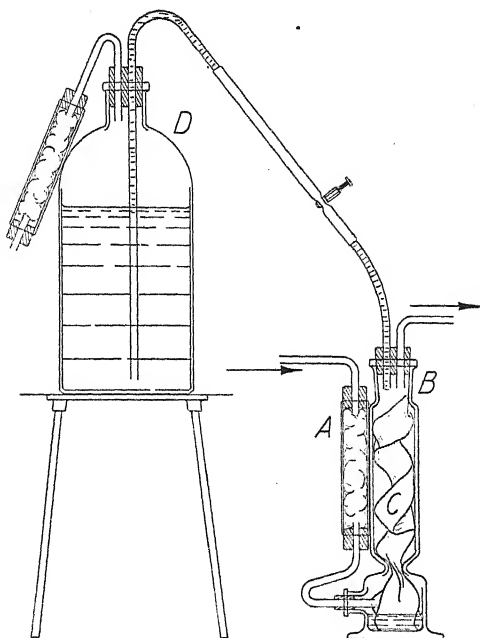


FIG. 5.—Apparatus for saturating air stream under sterile conditions: *a*, cotton filter; *b*, humidifier containing wet cloth (*c*); *d*, water supply. Outlet from humidifier leads to chamber which contains the experimental material.

towers containing a desiccating agent. Anhydrous magnesium perchlorate (anhydron) and anhydrous barium perchlorate (desic-chlora) are both suited to this purpose, because they absorb a relatively large proportion of water without liquefying. Anhydrous calcium chloride, phosphorus pentoxide, and silica gel are also used to desiccate air. Another way of drying air is to pass it over freezing coils. The moisture is precipitated from the atmosphere at a low temperature, and the air, when reheated, is almost dry. Air which

has been saturated at  $-10^{\circ}\text{C.}$ , for example, will have a relative humidity of about 9 per cent at  $25^{\circ}\text{C.}$  The dry and moist air streams may be combined in definite proportions by means of mixing valves, and if the temperature is controlled, any desired relative humidity can be obtained. Two precautions which must be taken in the application of this procedure are the careful regulation of the temperature and the use of accurate mixing valves.

#### INTERMITTENT VAPORIZATION

The most widely used physical method of humidity regulation is intermittent vaporization (1, 2, 3, 7, 8, 11, 14, 16, 21). This method is much more complicated and expensive than are the chemical procedures, but it has the advantage that chemical contamination of the cultured organisms is impossible, since only pure water is used. The employment of this method permits free circulation and renewal of air within humidity chambers of large capacity. When the desired moisture content of the atmosphere has been attained, humidification is interrupted, either electrically or mechanically, by a hygrostat contained within the humidity chamber. This instrument may operate in one of two ways, lineametrically or psychrometrically.

The regulating mechanism of a lineametric hygrostat is a material which increases in length owing to imbibition of atmospheric moisture. Human or animal hairs (14, 16, 21) are commonly used for the purpose, since they react quickly to small changes in relative humidity. Wood strips are used as the expanding medium in humidity-control apparatus designed for operation in large volumes, such as rooms. These instruments show a greater lag in reaction than do hair hygrometers. A simple hair hygrostat with electrical control can be constructed rather inexpensively (fig. 6). The hairs of the hygrostat actuate a lever at the end of which is a tungsten or platinum contact that dips into a mercury cup. The hygrostat works on a low-voltage battery circuit which operates the humidifier through a relay. One contact wire leads into the mercury cup and the other wire connects to the standard which supports the lever bearings. In using this apparatus it has been found that the surface of the mercury gradually oxidizes. The oxidized surface is easily skimmed off, however. Hygrometers of this type, unless carefully

designed, require occasional readjustment, because of the strain exerted on the hairs by the weight of the lever.

Construction of the psychrometric hygrostat is based on the cooling effect of evaporation from a moist surface (2). The evaporating surface is a linen cloth which is wrapped around the bulb of a mercury thermometer (fig. 7). The cloth is kept moist by absorption of distilled water through a wick from a reservoir placed below the thermometer bulb. The hygrostat is connected in series with the coil of a relay, the armature of which is in the humidifier circuit.

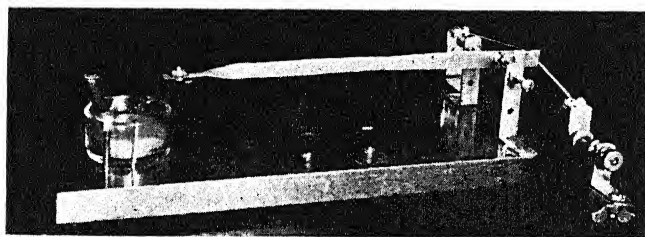


FIG. 6.—Hair hygrostat with electric contacts

One contact wire is sealed into the bulb of the hygrometer, and the other wire, made of platinum, extends down the neck to the surface of the mercury. Oxidation of the mercury surface, due to sparking, is avoided by filling the neck with castor oil or an oil of similar quality. The instrument is regulated to respond at a definite humidity by adjusting the wire in its neck to touch the mercury meniscus at a point corresponding to the desired reduction in temperature. A bimetallic-strip thermostat wrapped in cloth could be substituted for the mercury thermometer.

The two characteristics which determine the accuracy of a psychrometric hygrometer are sensitivity and lag. Sensitivity of the psychrometer depends on the ratio of the bore of the neck to the capacity of the bulb. Since the minimum bore is limited by the diameter of the wire, sensitivity can be increased only by using a bulb of large capacity. However, a large bulb will cause an excessive lag in the instrument. In the design of a hygrometer, therefore, a suitable compromise must be established between these two features.

The advantage of a psychrometric hygrometer is that no readjust-

ment is required after the first setting; it is necessary only to replenish the distilled water in the reservoir. This type of instrument possesses two undesirable features, however, which are not found in

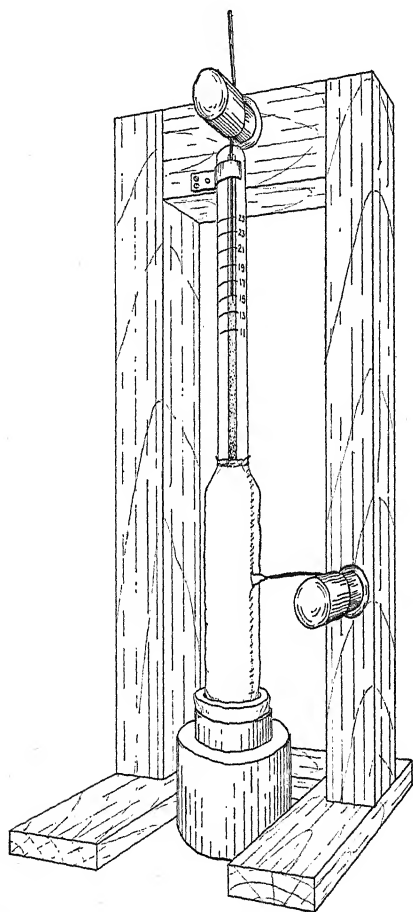


FIG. 7.—Psychrometric hygrometer with electric contacts.

the hair hygrometer. Firstly, continuous air movement is necessary for its correct operation, whereas the hair hygrometer operates as efficiently in still as in moving air. The second undesirable feature concerns the necessity of careful temperature regulation. The relative humidity secured by the use of this instrument depends on the difference between the predetermined temperatures of the wet bulb and of the air. If the air temperature varies, the difference between it and that of the wet bulb will be changed and a relative humidity other than the desired one will be attained. A change in relative humidity of approximately 6 per cent is induced by a  $1^{\circ}$  variation when the air temperature is  $25^{\circ}$  and the wet bulb is set at  $20^{\circ}$  C. The effect of variations in air temperature on the accuracy

of the humidity control depends on the temperature and relative humidity of the air within the chamber. At low temperatures and at high humidities, variations in temperature have the greatest effect. Temperature control must be accurate, therefore, if close humidity

regulation is to be attained with a psychrometric hygrostat. The lineametric hygrostat, on the contrary, operates independently of the air temperature since the hairs respond directly to changes in relative humidity.

In the case of control by intermittent vaporization, the air passes through the humidifier into the humidity chamber. When the humidity chamber is small, and when the volume and velocity of the incoming air are relatively great, the air may be humidified at room temperature by passive vaporization from a water surface. Under these conditions a simple humidifier is sufficient. Moistened cloth in a glass tower is suitable for this purpose. Humidification is stopped by interruption either of the air current or of the water supply (14, 21). When the volume of the humidity chamber is great, however, humidification at a temperature considerably above that of the air within the humidity chamber is desirable. Since the moisture content of the air at saturation is considerably increased by heating, the atmosphere of the humidity chamber can be humidified quickly by this means. Increasing the water-holding capacity of the humidifying air stream by heat is referred to in this paper as forced vaporization.

Several kinds of humidifiers operating on the principle of forced vaporization have been designed for use in incubating chambers (16, 21). A simple one, which was adopted in principle from that designed by STOUGHTON (16), has proved satisfactory. It consists (fig. 8) of a sheet-copper cylinder, 8 inches high and  $4\frac{1}{2}$  inches in diameter, which is fitted with a tight cover. An electric light socket and bulb are so secured in the cover that the bulb is suspended

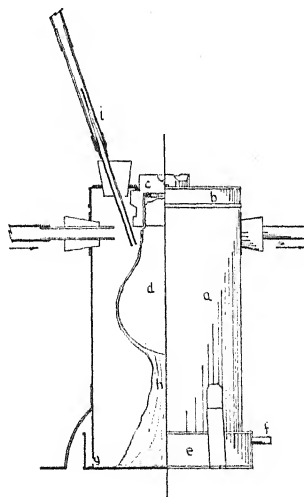


FIG. 8.—Humidifier of forced-vaporization type: *a*, exterior of metal cylinder; *b*, cover; *c*, electric light socket; *d*, electric bulb; *e*, tray; *f*, run-off spout; *g*, outlet from cylinder to tray; *h*, linen wick; *i*, constant-drip tube.

within the humidifier and the leads of the socket are external. The cylinder is set in a shallow copper tray, 5 inches in diameter and  $\frac{1}{2}$  inch in height, near the top of which is a run-off spout. One-quarter of an inch above the base of the cylinder is a  $\frac{1}{8}$ -inch hole which serves as a water outlet from the interior of the cylinder to the tray. A wick made of coarse linen is sewed around the electric light bulb and extends to the bottom of the humidifier. A constant-drip glass tube is set through the rubber stopper in the top cover of the humidifier

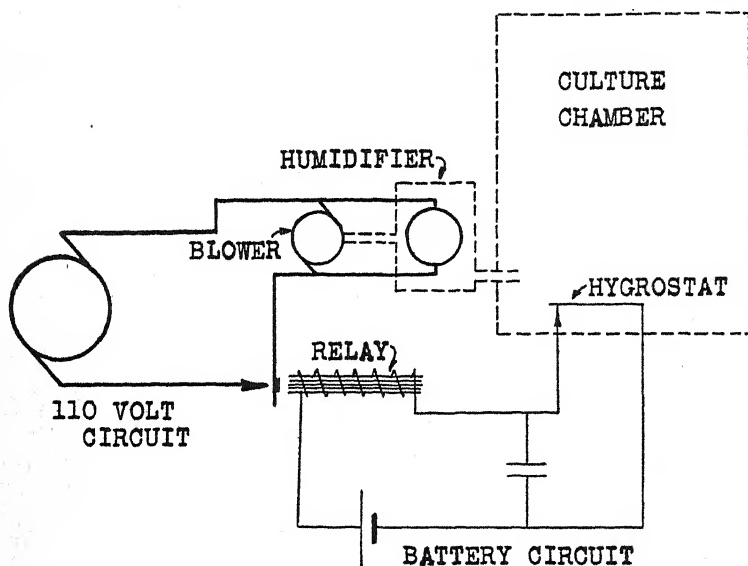


FIG. 9.—Electric hook-up for intermittent vaporization

so that the water drips on the wick near the base of the bulb. About  $1\frac{1}{2}$  inches below the top of the humidifier, two  $\frac{3}{4}$ -inch holes are drilled opposite each other. Air is blown into the humidifier through one of these openings and the other is connected by means of asbestos-wrapped glass tubing to the culture chamber. The air blower and the bulb of the humidifier are connected in series on a 110-volt circuit, which is controlled through the relay by the hygrostat (fig. 9). The size of the humidifier bulb is determined by the amount of vaporization desired; the larger the bulb the more rapid is the rate of humidification.

Although forced intermittent vaporization is the only practical means of humidity regulation in large chambers, the mechanical nature of the control equipment causes a perceptible lag in the instruments. The factors which cause this condition are such that the method gives good results only at ordinary incubating temperatures (fig. 11, *d*). Efficient control of the relative humidity at temperature extremes is difficult to obtain without the use of equipment which is considerably more complex than that just described. The reason for the impossibility of securing effective humidity control at extreme low and high temperatures is evident when some of the characteristics of atmospheric humidity are considered.

The relative humidity depends on three factors: absolute humidity, temperature, and atmospheric pressure. The latter factor is of such slight importance in humidity-control apparatus that it need not be considered further in the present discussion. The absolute humidity of a saturated atmosphere depends on the vapor pressure, which increases rapidly with a rise in temperature. The warmer the air, therefore, the more concentrated is the moisture in the atmosphere over a freely evaporating water surface. At  $0^{\circ}\text{C}.$ , saturated air contains about 4.8 gm. of water per cubic meter, at  $25^{\circ}$  it contains 23 gm., and at  $50^{\circ}$  almost 85 gm. (fig. 10). Relative humidity is a percentile expression of moisture content based on the absolute humidity of saturated air. In the case of saturated air at  $0^{\circ}\text{C}.$ , 1 per cent of the absolute humidity is 0.048 gm. per cubic meter. If it is assumed, for the sake of illustration, that the capacity of the humidity chamber is  $\frac{1}{2}$  cubic meter, then a 5 per cent variation in relative humidity represents a change of 0.08 gm. of water vapor. If humidifying apparatus similar to that described is used, this amount of moisture will be discharged into the atmosphere very rapidly. By the time the actuating part of the hygrostat reacts, the relative humidity might be increased by 10 or 20 per cent. With the common methods of control, therefore, it is impossible to humidify the air slowly enough at low temperatures to prevent a considerable lag in the response of the hygrostat. The higher the temperature, the less important is the lag of the hygrostat. This is due to the larger quantity of vapor required to effect a unit change in relative humidity. The hair hygrostat and humidifier which have been described



function acceptably well above  $15^{\circ}\text{C}$ . Below this temperature, attempts to secure accurate humidity control were unsuccessful.

At high temperatures, the difficulty in securing effective humidity control is due not to the hygostat, but rather to the humidifier. The moisture holding capacity of the atmosphere increases rapidly

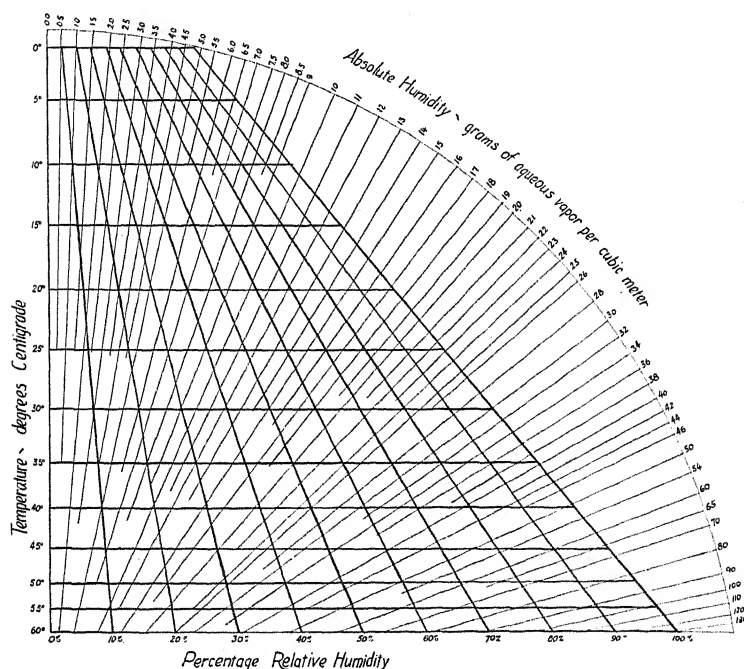


FIG. 10.—Temperature-absolute humidity-relative humidity diagram. Data calculated from MILLIKAN (10).

at the higher incubating temperatures. A slight variation in temperature will therefore induce a considerable change in moisture holding capacity of the air. If it is assumed, for the sake of illustration, that the relative humidity in a  $\frac{1}{3}$  cubic meter chamber is to be held at 80 per cent, it would be necessary in order to counteract the effect of a temperature change from  $58^{\circ}$  to  $62^{\circ}\text{C}$ . to discharge an additional 6 gm. of water into the air. The rate of humidification with the type of apparatus described is too slow to counteract the effect of this  $4^{\circ}$  temperature rise. As the temperature falls, the rela-

tive humidity may go as much as 10 per cent above the desired amount (fig. 11, *a*). The incubating temperature which is employed in culture studies of fungi does not generally exceed 40° C., however, and is usually about 30°. At these temperatures, humidity control

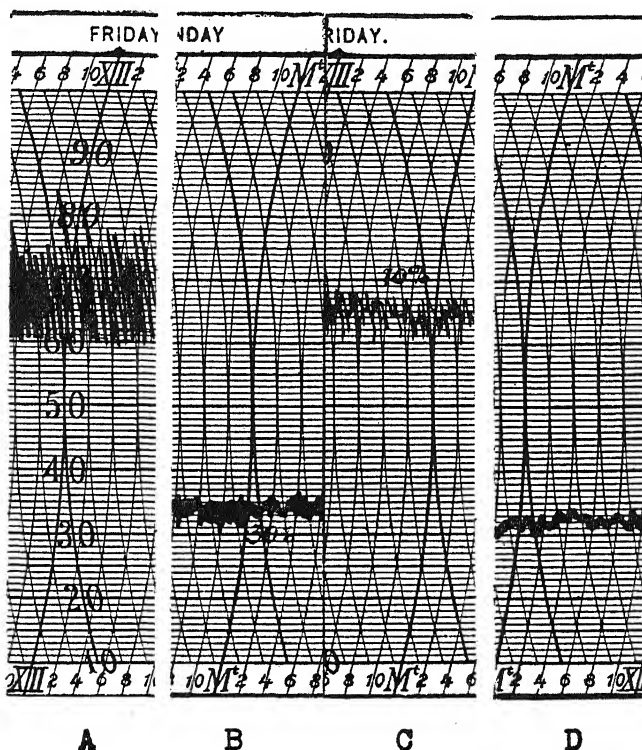


FIG. 11.—Hygrograph records obtained in chambers humidified by intermittent vaporization with hair hygrostat and humidifier of forced-vaporization type: *a*, 67 per cent relative humidity at 52° C. showing excessive variation at high temperatures; *b*, 33 per cent relative humidity at 52° C.; *c*, 67 per cent relative humidity at 38° C. (hygrostat in latter chamber only, humidified air stream being divided equally into the two chambers); *d*, effective control at 25° C. and 33 per cent relative humidity.

with the described apparatus is successful since the rate of vaporization is sufficiently slow to permit the efficient operation of the hygrostat, and is rapid enough to counteract the effect of temperature variation.

When humidity chambers are operated in sets, it has been found possible to simplify the control apparatus considerably. If within two chambers of equal volume the temperature is held at different levels and the humidified air stream is divided, half being blown into each chamber, the relative humidities obtained will be an inverse function of the temperatures. For example, if a hygrostat in one chamber which is operated at  $38^{\circ}$  C. is regulated so as to secure a relative humidity of 67 per cent, the relative humidity in the other chamber can be calculated from the temperature. Since equal amounts of moisture are discharged into both chambers, the absolute humidity in each will be the same, 30.6 gm. per cubic meter (fig. 10). If the second chamber is operated at  $52^{\circ}$  C., the relative humidity in it will be 33 per cent. In tests which were run under these conditions, satisfactory humidity regulation was obtained in both chambers, although only the one set of control apparatus was used (fig. 11, *b*, *c*).

### Summary

1. Atmospheric humidity can be controlled in culture studies by quantitative and qualitative chemical methods, and by physical methods involving continuous and intermittent vaporization.
2. The quantitative method of chemical control is convenient for small closed chambers. It is easily set up and is inexpensive.
3. The method of qualitative chemical control is used for the regulation of humidity in both closed and fresh air chambers. This method is simple, and requires very little equipment and care.
4. Physical control by means of continuous vaporization is useful to secure a saturated atmosphere. To secure relative humidities intermediate between 0 and 100 per cent, the additional apparatus required generally makes the method impractical for use in culture studies.
5. Intermittent vaporization is the most useful method for large chambers. Control is by means of a hygrostat and a humidifier.
6. Hygrostats are of two types, lineametric and psychrometric. The first acts independently of temperature variation, but requires frequent readjustment unless well designed. The second can be used only when the air temperature is accurately controlled. Under these

conditions, it is more satisfactory than the lineametric hygostat since readjustment is not required.

7. Humidifiers operate by either passive or forced vaporization. Those which operate on the principle of passive vaporization are used with chambers of small capacity and at low temperatures; those of the forced vaporization type are employed to humidify large quantities of air and for control at high temperatures.

8. Humidity control by physical methods is effective between temperatures of 15° and 40° C. Beyond these extremes, technical difficulties result in decreased accuracy of the humidity control.

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# ANATOMICAL STRUCTURE OF STEMS IN RELATION TO THE PRODUCTION OF FLOWERS

OCRA CHRISTINE WILTON AND R. H. ROBERTS

(WITH FORTY-THREE FIGURES)

## Introduction

In connection with the problem of reproduction in higher plants, it is important to determine whether the phenomenon of reproduction has a common physiological basis for a wide range of plants or whether each variety has its own particular physiological condition accompanying the production of blossoms. Although wholly different external environments are required to induce blossoming in different species and varieties as illustrated by long- and short-day plants (6, 7), it is possible that a common internal condition may accompany reproduction. Should this be demonstrated, it would greatly aid in the solution of the important practical and technical question of why plants blossom.

LOEW (10), FISCHER (5), and others have considered this point when proposing a nutritional basis for sexual reproduction. The suggestion of KRAUS and KRAYBILL (9) that fruitfulness depends upon the relationship between the carbohydrate and nitrogen composition of the plant also proposes a physiological basis for fruitfulness. FINCH (4), NIGHTINGALE (12), and ROBERTS (13) have presented data which were interpreted as supporting the carbohydrate-nitrogen hypothesis, while ARTHUR and coworkers (1) and WORK (17) interpret their data as contradicting the existence of such a relationship. There is an ever increasing body of evidence to show a positive correlation between the character of growth of a plant and its reproductive condition (14).

Although chemical studies to date have apparently left the matter unsettled, it is possible that other methods of approach can provide more definite data. A study of carbon dioxide exchange in a number of species has indicated that a common physiological condition of the plant accompanies reproduction (15, 16). The present report

gives the results of observation made in a number of species to determine the relation between certain anatomical characteristics in the stem and the production or non-production of flowers.

Although anatomical studies have been made upon plants grown under different environmental conditions, there has been little emphasis upon the possible correlation between anatomy and reproduction. DEATS (3) reports that tomato and pepper plants which produced few or no flowers in a short-day environment have more pith and less xylem and bast than plants grown in a long-day environment. The walls of the cells of the xylem and bast are thinner, and although there are fewer large vessels, their average diameter is larger in the non-flowering plants than in comparable cells and tissues of the plants which produced flowers. FINCH (4) notes that the xylem of the fruitful growth of biennially bearing apple trees shows the most summer wood, including a relatively large number of parenchymatous cells. The unfruitful terminal growth has the least amount of summer xylem and the proportion of fibers to parenchyma is high.

### Materials

In attempting to determine a possible correlation between the anatomy of stems and the production or non-production of flowers, a wide range of species was examined during a period of five years. A comparison was made of the flowering and non-flowering stems of twenty-eight species of plants belonging to fourteen families, as follows:

Amaranthaceae: *Amaranthus retroflexus* L.

Aizoaceae: *Tetragonia expansa* Murr.

Caprifoliaceae: *Sambucus canadensis* L.

Cruciferae: *Cheiranthus cheiri* L., *Mathiola incana* R. Br.

Geraniaceae: *Pelargonium domesticum* Bailey, *P. peltatum* Ait.

Linaceae: *Linum usitatissimum* L.

Euphorbiaceae: *Euphorbia pulcherrima* Willd., *Ricinus communis* L.

Begoniaceae: *Begonia semperflorens* Link and Otto.

Onagraceae: *Clarkia elegans* Dougl., *Fuchsia hybrida* Voss.

Apocynaceae: *Vinca major* L.

Labiatae: *Coleus blumei* Benth., *Salvia splendens* Ker.



Solanaceae: *Datura stramonium* L., *Lycopersicon esculentum* Mill., *Nicotiana tabacum* L., *Petunia hybrida* Vilm., *Schizanthus wisetonensis* Low., *Solanum tuberosum* L.

Scrophulariaceae: *Antirrhinum majus* L.

Compositae: *Ageratum conyzoides* L., *Calendula officinalis* L., *Chrysanthemum morifolium* Ram. var. Lillian Doty, *Cosmos sulphureus* Cav. var. Klondike, *Zinnia elegans* Jacq.

Except in the case of flax, the plants from which the material was obtained were grown under controlled conditions in the greenhouse, photoperiod being the variable factor used to modify or regulate the production of flowers.

The flax was grown in the garden during the summer of 1931. Prior to flower production, samples of stems were taken which were classed as non-flowering. Later, when flowers were present, samples of "flowering" stems were taken. At this time, one row of plants was shaded with 8-oz. burlap and given a mixture of ammonium sulphate and sodium nitrate (two-thirds  $(\text{NH}_4)_2\text{SO}_4$  and one-third  $\text{NaNO}_3$ ), applied broadcast as nearly uniformly as possible at the rate of 500 pounds per acre. These plants became rather weakly vegetative and produced new branches. After a time the untreated plants also began a "second growth," as indicated by the development of many new branches. Samples of the stems were taken from both groups of plants below the point of origin of the new growth.

The remaining species are represented by samples of stems of flowering and non-flowering plants which were growing in the University greenhouses and gardens. This type of material was used for the preliminary study, and in all instances the observations indicated that the anatomical characteristics were correlated with the reproductive stage rather than with the cultural conditions or age of plant. The present report, however, is limited to material from the controlled plants.

In addition to samples from strictly flowering and non-flowering plants, samples were taken of the stems of plants of cosmos, chrysanthemum, and zinnia on which flower buds were first appearing. "Budding" plants were considered a transition stage from a non-reproductive to a distinctly reproductive type of growth.

Plants which had changed from a reproductive to a non-produc-

tive type of growth, either by inducing abortion of blossoms or by the production of vegetative shoots following the development of flowers, were also examined. The abortion of flower buds was induced in plants of cosmos by the transfer of budding plants from a short- to a long-day environment, while the opposite change from a long- to a short-day environment was necessary to induce the abortion of flower buds on plants of petunia. Flowering plants of poinsettia which were transferred to a long-day environment developed vegetative shoots. Samples were taken from the internode below the topmost vegetative shoot.

Two species, cosmos and *Ricinus*, were induced to produce a second set of flowers following a period of vegetative growth. Samples of this material were taken as an additional means of checking how closely the anatomy might be associated with the external character of growth throughout a flowering phase, a non-flowering phase, and a second flowering phase.

It is commonly stated that the usual woodiness of plants at the time of flowering is a result of the seasonal maturity which generally accompanies the production of flowers or is in fact the result of flowering itself. In order to determine whether or not this condition of "maturity" is a result of blossoming or one which precedes fruiting and is inherent in the reproductive stage, plants of cosmos and petunia were disbudded in order to prevent the development of flowers. It seemed that if deflorated plants developed a "mature" structure in contrast to the situation in vegetative non-blossoming plants, this would be evidence of a positive relation between stem structure and reproductive condition. The disbudding procedure consisted of the removal of flower buds about twice a week, while they were very small but after they were sufficiently differentiated to be distinguished from any foliar primordia which might be present.

### Methods

Samples of flowering stems were taken from the second internode below terminal inflorescences as in cosmos. Where there was a vegetative growing point above the flower, as in petunia, samples were taken from the fourth internode below the youngest flower. Samples of non-flowering stems were taken from the fourth elongated inter-

node from the tip of the stem, a region in which secondary tissues are being differentiated.

Preliminary observations were made from free-hand sections of the stems. The sections were stained with an aqueous solution of iodine-potassium iodide to which had been added an equal volume of 1 per cent eosin in 95 per cent alcohol. This combination gives a better distinction between wall and cell content in material which contains little or no starch than is obtainable by the use of iodine-potassium iodide alone. The sections were then mounted on slides in glycerin containing iodine-potassium iodide.

For permanent mounts, the samples of stems were fixed in formalin-acetic-alcohol (6 cc. formalin, 2 cc. glacial acetic acid, and 100 cc. of 50 per cent alcohol). Both ethyl alcohol and butyl alcohol were employed at different times as dehydrating agents. The fixed material was imbedded in paraffin and cross sections 15–20  $\mu$  in thickness were made. In the earlier work, safranin and light green were used for staining the sections but later a combination of aqueous crystal violet and erythrosin in clove oil was found to be very satisfactory for differentiation of the tissues. These last two stains were less satisfactory for photomicrographs, however, and when photomicrographs were desired the sections were stained with cotton red alone.

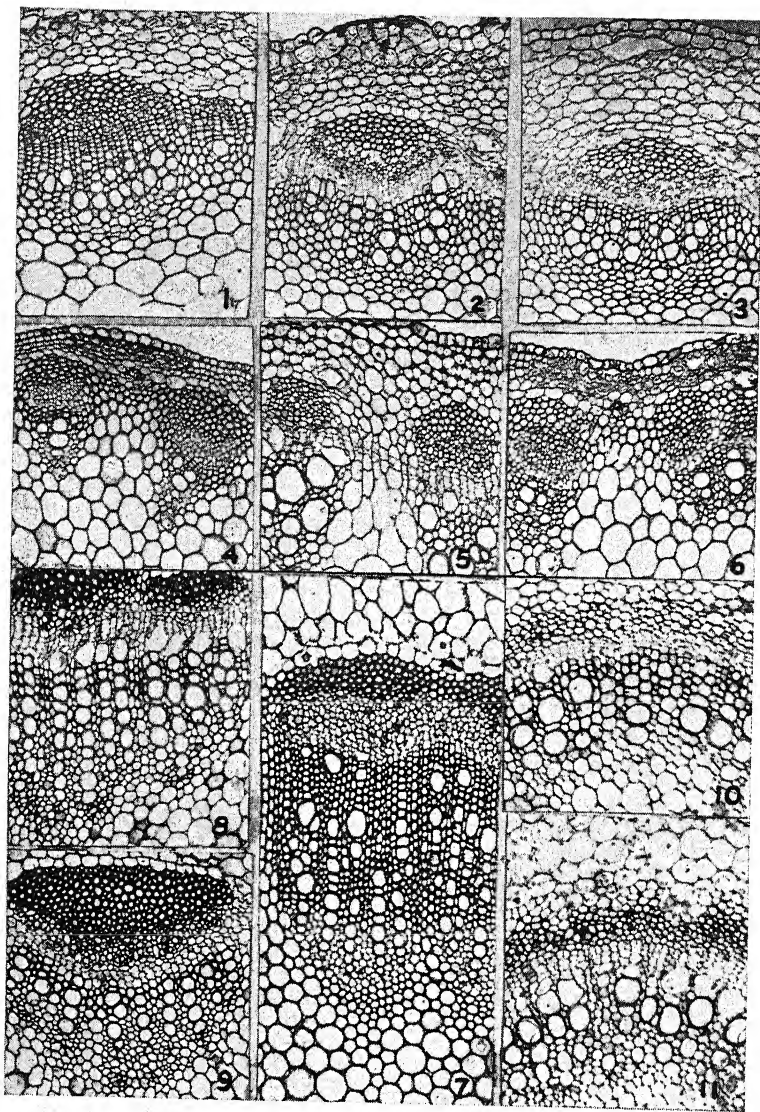
### Observations and discussion

A number of questions arose in the course of these studies. The first one to be considered may be stated thus: Are there consistent anatomical differences in the stems of plants producing flowers and of plants not producing flowers?

It was found that there were characteristically different conditions in the flowering and non-flowering stems of the various plants examined. These differences were more pronounced in some species than in others. *Chrysanthemum* will be used as an illustration of a plant having a markedly distinctive anatomical structure in the flowering stem (figs. 1, 14).

There appear to be no meristematic cells present in the cambial region.<sup>1</sup> All the cells of the pericycle and phloem, except a few of the

<sup>1</sup> The leaf traces commonly have a relatively inactive cambium in both flowering and non-flowering stems. These have not been included in the characterization of the anatomy of the various samples.



FIGS. 1-11.—Photomicrographs of portions of transverse sections of stems: Figs. 1-3, chrysanthemum (1, from a flowering plant; 2, from a non-flowering plant; 3, from a budding plant). Figs. 4-6, cosmos (4, from a flowering plant; 5, from a non-flowering plant; 6, from a budding plant). Figs. 7-9, zinnia (7, from a flowering plant; 8, from a non-flowering plant; 9, from a budding plant). Figs. 10-11, salvia (10, from a flowering plant; 11, from a non-flowering plant).  $\times 100$ .

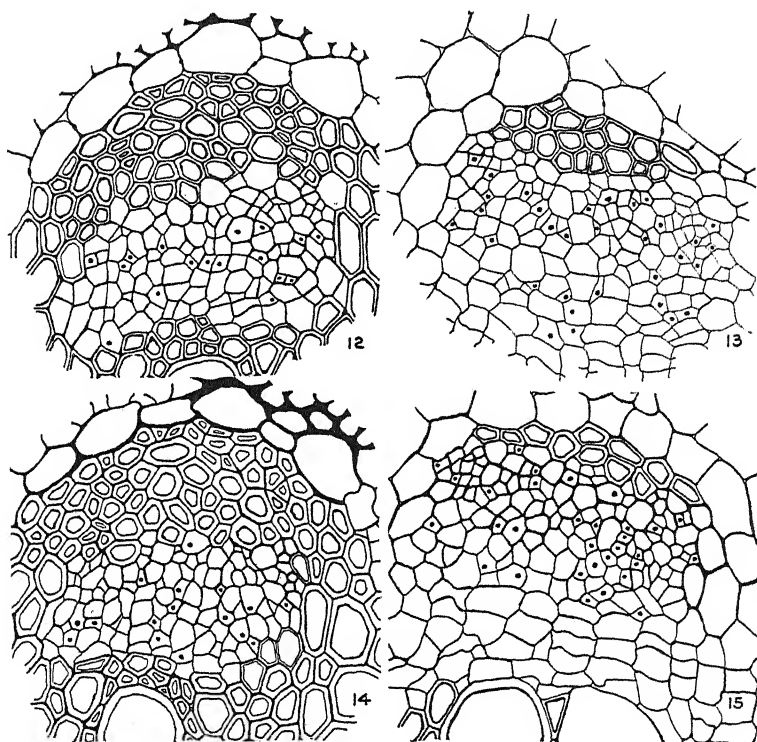
sieve tubes and companion cells, have become thick-walled and are stained by the basic dye, crystal violet. The last formed secondary xylem consists entirely of small, thick-walled, usually oblong elements which appear to be of one type when seen in transverse section. When these elements are examined in longitudinal sections they are found to be of at least two types, some having tapering end walls while others have almost square end walls. All are more or less conspicuously pitted. The xylem and entire perimedullary zone are also stained by crystal violet.

In contrast to these characteristics, the comparable internode of a non-flowering stem of chrysanthemum (figs. 2, 15) shows an active cambial zone four to six cells in width, which is composed of typical thin-walled elements. Numerous xylem vessels as well as companion cells and sieve tubes are in process of differentiation. Fewer of the cells of the pericycle have thickened walls than is the case in the flowering stem, although the pericyclic fibers have become fully as thick-walled as corresponding ones in the flowering stem. In the non-flowering stem, only the walls of the pericyclic fibers and the fibers of the phloem and the xylem vessels are stained by crystal violet, the remaining cells being stained by erythrosin. The smaller amount of tissue in the non-flowering stem in contrast to that of the flowering stem which is stained by the basic dye is to be noted.

Sections from stems of plants of chrysanthemum which were just beginning to develop flower buds show a structure intermediate between that of the flowering and non-flowering stems (fig. 3). There is a cambial zone present but it averages only two cells in width. As is characteristic of the flowering stem, small xylem elements, which are oblong in section view, are being differentiated on the inner side of the cambium.

The anatomical characteristics of the flowering, budding, and non-flowering stems of cosmos closely parallel those observed in the comparable stems of chrysanthemum. Both species belong to the same photoperiodic group and require less than fourteen hours of light per day for the production of flowers. The only indication of cambium in the flowering stem of cosmos (figs. 4, 12) is the presence of a few meristematic cells, generally three or four per bundle. The secondary xylem adjacent to the cambium is made up of small,

thick-walled cells. The walls of the cells of the pericycle as well as of those of the perimedullary zone have become thickened. Many of the older cells of the phloem have also become relatively thick-walled. The non-flowering stem of cosmos (figs. 5, 13) has a relatively wide cambial zone, and in the xylem numerous vessels of large



FIGS. 12-15.—Camera lucida drawings of portions of bundles from flowering and non-flowering stems: Figs. 12, 13, cosmos showing relatively larger elements in youngest phloem of non-flowering stem (fig. 13) when compared with those of flowering stem (fig. 12). Apparent absence of meristematic cells in cambial zone seen in fig. 12. Figs. 14, 15, chrysanthemum showing characteristics similar to those noted for cosmos in figs. 12 and 13.

lumen and many thin-walled parenchymatous cells are in process of differentiation. Both the interfascicular pericycle and the perimedullary zone are composed of relatively thin-walled cells. The vascular bundles in the stems of the budding plants of cosmos (fig. 6) show

a narrow fascicular cambial zone, and sometimes there is still a suggestion of interfascicular activity. The cells of the pericycle and primedullary zone have, in general, thinner walls than comparable cells in the flowering stems. The small secondary xylem elements which are characteristic of flowering stems are again present.

Sections of the phloem of flowering and non-flowering stems of cosmos (figs. 12, 13) show that the cells of the secondary phloem of the non-flowering stem are relatively large in transverse section compared with those of the primary phloem, and the cells of the secondary phloem in the flowering stem appear to be little larger than the cells of the primary phloem. The whole region in the flowering stem has cells with thicker walls. Sections from stems of chrysanthemum show similar anatomical characteristics (figs. 14, 15).

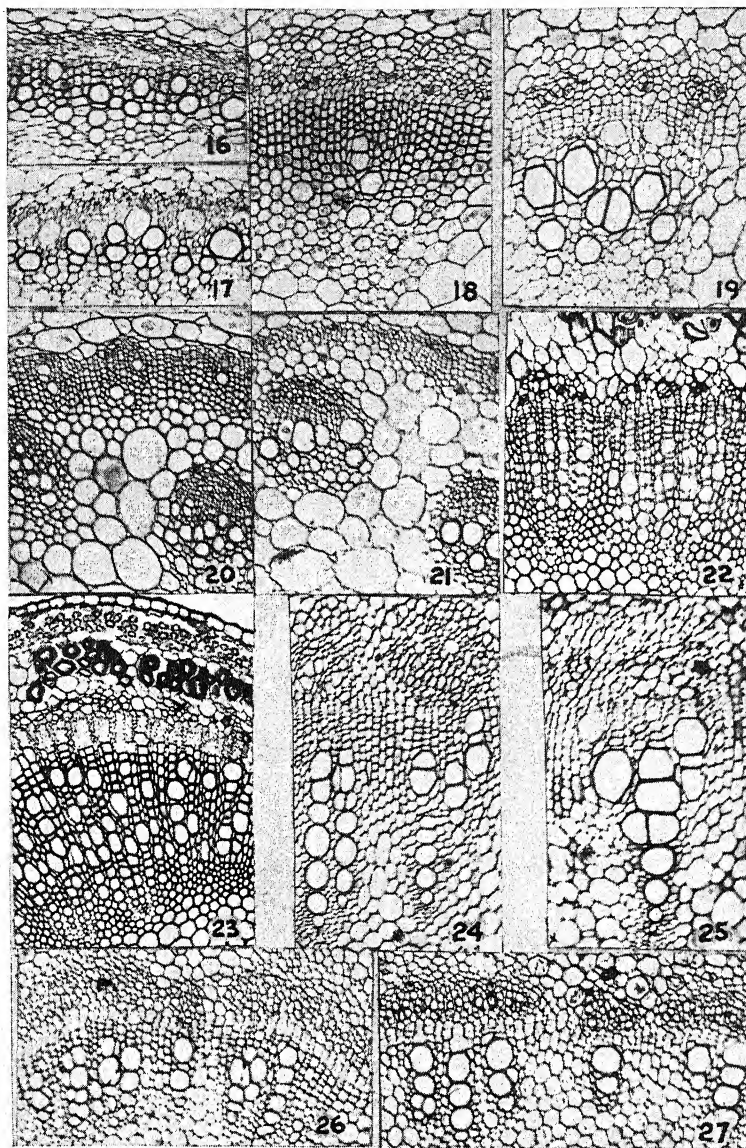
In salvia, another short-day species, the structure of the flowering stems is somewhat less distinctive than that of chrysanthemum and cosmos. The flowering stems show a relatively wide cambial zone, but the last formed secondary xylem consists of small thick-walled cells with only a few large vessels in process of differentiation (fig. 10). The non-flowering stem of this species has a distinctly different appearance, however, with a wider, more active cambial zone than the flowering stem; and the secondary xylem is composed of thin-walled parenchymatous cells and numerous large vessels (fig. 11).

Sections from flowering and non-flowering stems of *Amaranthus retroflexus* (figs. 20, 21) and poinsettia (figs. 26, 27) also have anatomical characteristics which have been described as typical of the respective types.

A short-day environment was needed to induce blossoming of the plants already discussed. Examination of a long-day and an indeterminate (6) type shows that the anatomical condition is not related to the photoperiod needed to initiate blossoming, but to the reproductive condition of the plants. This fact is illustrated by the sections of *Zinnia elegans* (figs. 7-9), New Zealand spinach (figs. 16, 17), *Coleus blumei* (figs. 18, 19), and flax (figs. 22, 23).

The flowering and non-flowering stems of *Ricinus* (figs. 24, 25) are of particular interest because the phloem elements as well as the xylem elements seem to show a marked correlation with the character of growth. The phloem elements which are in process of differ-



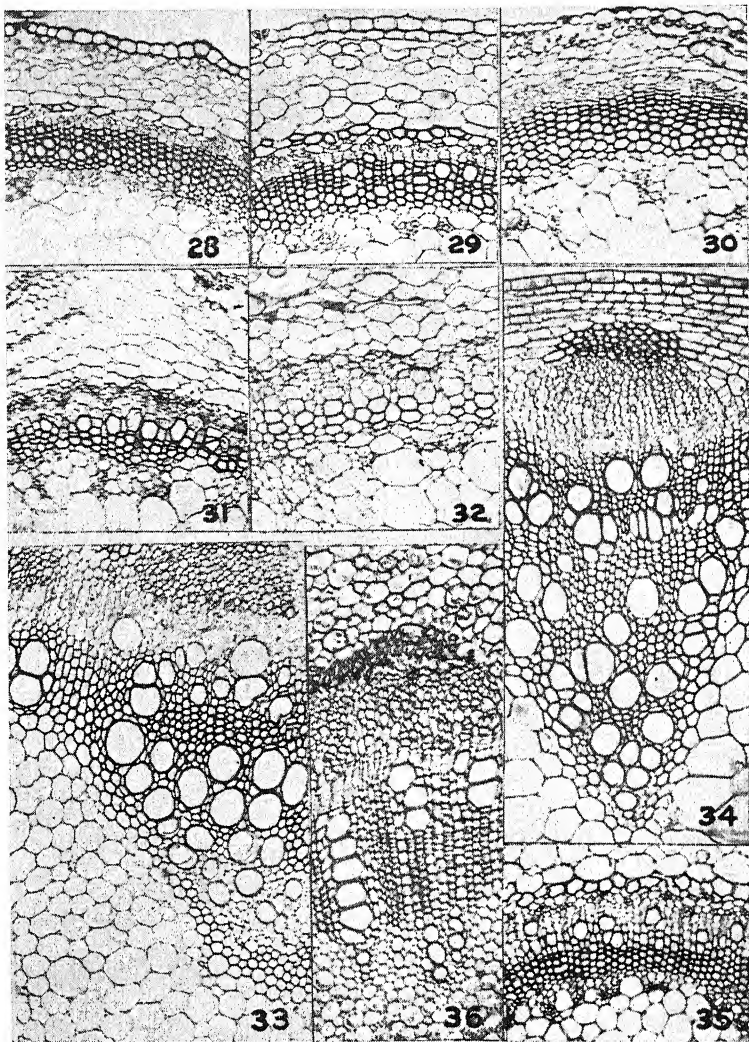


FIGS. 16-27.—Photomicrographs of portions of transverse sections of stems: Figs. 16, 17, New Zealand spinach (16, from a flowering stem; 17, from a non-flowering stem). Figs. 18, 19, coleus (18, from a flowering stem; 19, from a non-flowering stem). Figs. 20, 21, *Amaranthus retroflexus* (20, from a flowering stem; 21, from a non-flowering stem). Figs. 22, 23, flax (22, from a flowering stem; 23, from a non-flowering stem). Figs. 24, 25, *Ricinus* (24, from a flowering stem; 25, from a non-flowering stem). Figs. 26, 27, poinsettia (26, from a flowering stem; 27, from a non-flowering stem).  $\times 100$ .

entiation in the non-flowering stem are relatively large in cross-sectional diameter, while in the flowering stem a few large elements have differentiated but the ones adjacent to the cambial zone are small in diameter.

Sections from the stems of plants of petunia in which flowering was only slightly delayed by a short-day environment as compared with plants which were grown with sixteen hours of light per day have an anatomical structure which suggests the flowering condition, even though flowers had not yet been produced (figs. 28, 29). There is a cambium present in both instances although it appears to be slightly less active in the flowering than in the non-flowering stem. In figure 32, however, is shown a section from an extremely vegetative, non-flowering plant of petunia which had been grown during the short days of November and December. In this case the structure does not resemble that of a flowering stem, but has the anatomical characteristics of a non-flowering stem. The differences between the flowering and non-flowering stems of plants of *Schizanthus* are greater than those found in petunia (figs. 30, 31).

Before summarizing the results of the observations reported thus far, attention is called to an interesting condition which has a bearing upon the interpretation of the data. This is the extent to which the primordia are utilized in flowering and might be called the degree to which a plant becomes reproductive. Those species, as cosmos and amaranthus, which differentiate flower buds at most of the growing points, usually die following fruiting. In contrast to these, species such as petunia continue to grow vegetatively while flowers are being produced. Flax attains a degree of reproductiveness intermediate between that of cosmos and petunia, in that numerous flowers are produced for a time during which there is little or no vegetative growth, but later there may be a renewal of vegetative activity. In this study it has been observed that cambial activity seems to be distinctly correlated with degree of flowering. With plants on which most of the primordia are reproductive, there is almost a complete absence of cambium. On the other hand, plants which make some vegetative growth while flowering, or which again become vegetative readily, have always shown more or less cambium although not necessarily in an active condition. This question of the relation of



FIGS. 28-36.—Photomicrographs of portions of transverse sections of stems: Figs. 28, 29, *petunia* (28, from a flowering stem; 29, from a non-flowering stem). Figs. 30, 31, *Schizanthus* (30, from a flowering stem; 31, from a non-flowering stem). Fig. 32, *petunia*, from a very vegetative, non-flowering plant. Figs. 33-36, sections taken below aborted floral buds (33, portion of section of *cosmos* taken from internode below aborted bud shown in fig. 37 showing zone of small thick-walled xylem elements followed by zone of relatively thin-walled elements and numerous vessels and active cambium). Fig. 34, section of *cosmos* taken below aborted floral buds of an old plant. Fig. 35, *petunia*. Fig. 36, *poinsettia*.  $\times 100$ .

cambial activity to renewal of vegetative growth following flowering as well as a consideration of the cambial condition at different levels along stems with many internodes is being made the subject of a separate paper.

Regarding a possible relation of anatomy to reproduction, the evidence is interpreted as showing a positive correlation, since flowering and non-flowering stems of a variety have certain distinguishing characteristics regardless of the age of the plant or the environmental conditions necessary to induce blossoming. The anatomical characteristics observed in the flowering and non-flowering stems may be summarized as follows: (1) The cambium of the flowering stem shows little if any activity while the non-flowering stems have a relatively wide, active cambial zone. (2) The secondary xylem lying adjacent to the cambium in the flowering stems of all the species studied consists mainly of small thick-walled elements and relatively few vessels as seen in section view, while in the non-flowering stem it consists characteristically of thin-walled parenchyma and more numerous vessels. (3) A greater number of the cells of the pericycle and of the perimedullary zone have thicker walls in the flowering than in the non-flowering stem. (4) In the flowering stem, the pericycle, perimedullary zone, and certain elements of the xylem tend to stain with basic dyes to a greater extent than do the comparable regions and elements in the non-flowering stem. (5) The phloem region of the flowering stems has relatively thick-walled cells as compared with that of the non-flowering stems.

The second question to be considered is: Will the elements produced by cambial activity at the time a vegetative non-flowering period of growth occurs following the production of flowers be characteristic of a flowering stem or will they resemble those of a non-flowering stem?

Figure 37, right, illustrates the type of floral abortion which occurs when budding plants of cosmos are transferred from a short- to a long-day environment. The time required to effect the change varies with the degree of differentiation of the flower buds at the time the photoperiod is altered. Plants which have well developed buds or open flowers when placed in a long-day environment may continue to produce blossoms for varying lengths of time and finally die in-

stead of renewing vegetative growth. The anatomical structure in the internode below the peduncle of the aborted bud is shown in figure 33. There is a wide cambial zone similar to the vegetative stem of figure 5 and numerous vessels and thin-walled xylem parenchyma have been recently differentiated. Previous to this there has been a period in which the xylem elements were practically all small thick-walled cells such as are found in the stems of flowering plants (fig. 4). Figure 34 illustrates the situation in one of a set of

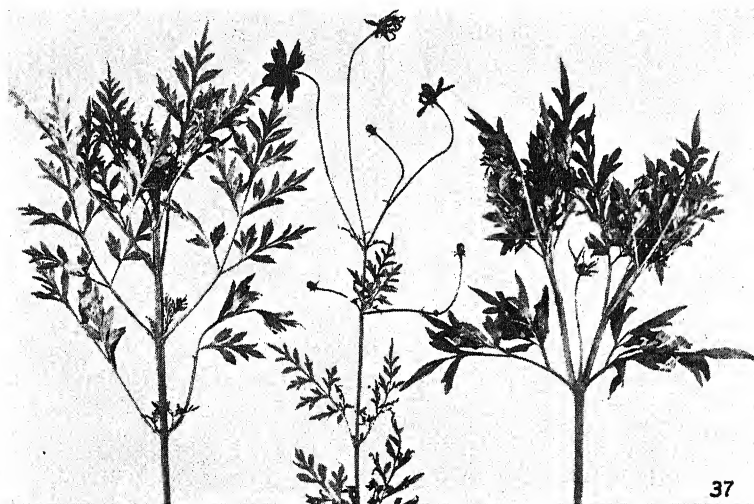


FIG. 37.—Terminal internodes of three plants of cosmos. Left to right: non-flowering plant grown in long-day environment; flowering plant grown in short-day environment; plant bearing aborted floral bud, grown first in short- and then in long-day environment.

plants in which the flowers had been permitted to develop to a more advanced stage before they were transferred to a long-day environment, and consequently required a greater time before there was a return to a vegetative type of growth. In this case there is also an active cambium. However, the change in type of xylem tissue from that characteristic of flowering to that of non-flowering stems has been by a less abrupt transition than in figure 33.

The anatomy found in stems of plants of petunia (fig. 35) on which abortion of floral buds had been induced is similar to that described for cosmos. There is an active cambium, while the last formed xylem

consists of numerous large vessels and thin-walled elements. This type of tissue has succeeded a zone in which the elements resemble those of the flowering stem (fig. 28).

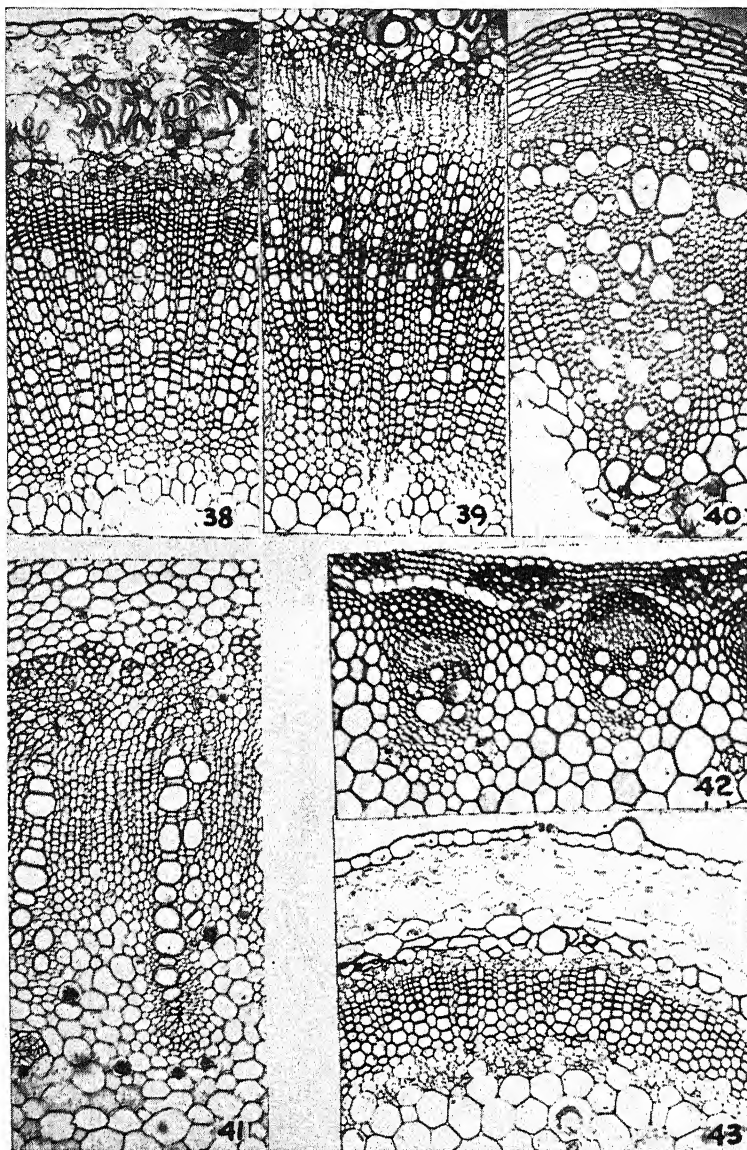
It should be especially noted that the condition of the cambium and the character of the xylem which are associated with the abortion of flowers and the return to a vegetative type of growth are the same for both cosmos and petunia, although a long-day environment was required for cosmos while a short-day environment was necessary for petunia.

Another case in which the anatomical character shifted with the change from a reproductive to a vegetative type of growth can be seen in poinsettia (fig. 36). Since this is a short-day plant, the return to a non-flowering character of growth was induced by a long-day environment similar to that required for cosmos but opposite to that used for petunia.

In the case of flax, the plants became vegetative after mature seeds had been produced. Figure 38 shows a section taken from the stem below the second growth of a plant which had been shaded and was weakly vegetative. In this material the character of the last formed xylem is similar to that which is associated with vegetative type of growth, but the cambium appears to have been only slightly active. Sections from the unshaded plants which were growing vigorously after producing seeds (fig. 39) show a wide cambial zone and there is evidence of considerable cambial activity. The xylem elements being differentiated are also similar to those which are characteristic of non-flowering plants (fig. 23).

Plants of cosmos and *Ricinus* which were producing a second series of blossoms following an intervening period of vegetative growth have stems which show anatomical changes that correspond to the changes in reproductive condition (figs. 40, 41); that is, there is a zone in the xylem in which the elements are larger in diameter and have thinner walls, between an earlier and a present region in which the cells are small and thick-walled as is typical of plants in a flowering condition. The *Ricinus* stem has only a few newly formed cells of the "reproductive" type. It also shows a marked alteration in the character of phloem cells, corresponding to the changes in character of growth from reproductive to vegetative and again to reproductive.

Similar differences in structure have been reported by HAWKINS



FIGS. 38-43.—Fig. 38, section from stem of flax plant which had become weakly vegetative after being shaded with burlap and given a treatment of  $\text{NaNO}_3$  and  $(\text{NH}_4)_2\text{SO}_4$ . Fig. 39, section from stem of flax plant which was making a second period of vegetative growth. Fig. 40, portion of section of cosmos plant which had produced a second set of flowering branches after period of vegetativeness. Fig. 41, *Ricinus*, same as fig. 40 except that second flowering phase was just beginning when sample was collected. Fig. 42, section from stem of disbudded cosmos plant. Fig. 43, same of petunia.  $\times 100$ .



*et al.* (8) in work on the physiological factors affecting the fruiting of cotton. They noted that rapid vegetative growth, as expressed through the formation of large thin-walled cells in the secondary xylem tissue of the stem, is accompanied in a general way by an increased shedding of bolls. Photographs of sections of such stems of cotton which accompany the report of HAWKINS indicate a change in the structure of the xylem from one resembling "summer wood" to one resembling "spring wood."

Thus in answer to the question of whether or not the elements produced by cambial activity when a vegetative non-flowering type of growth follows the production of flowers will resemble those of a flowering or of a non-flowering stem, the observations upon the five species examined, cosmos, poinsettia, petunia, flax, and *Ricinus*, as well as the report of HAWKINS, indicate that there is a correlation between the anatomy of the stems and the reproductive character of growth of the plants.

The third question for consideration in this study may be stated: Is the type of structure which is characteristic of flowering stems the result of the production of flowers or of a physiological condition which initiates both the production of flowers and the anatomy?

Evidence upon this question was secured by disbudding plants in the early stages of flower formation. Sections of the stems of disbudded plants of cosmos have a structure which resembles that of a flowering stem (fig. 42). They have an apparently inactive cambium and the last xylem elements are small, thick-walled, and tangentially flattened. However, the cells of the perimedullary zone, pericycle, and xylem have even thicker walls than are generally seen in a flowering stem. Sections from disbudded stems of plants of petunia show thick-walled cells in these same regions, although the thickness of the walls is, in general, somewhat less noticeable than in cosmos (fig. 43). The observations made on cosmos and petunia agree in general with those reported by MIRSKAJA (11), who removed the flower buds of *Mirabilis jalapa*, *Zinnia elegans*, and *Ageratum mexicanum*. *Zinnia* and *ageratum* showed a striking production of lignified material, a phellogen, and an endodermis with Casparian strips. In the present study, sections from stems of disbudded plants of cosmos show that all of the tissues except the central pith cells, cor-



tex, and the youngest phloem elements have markedly thick-walled cells which stain with the basic dyes. The development of an endodermis with Casparian strips seems to be a characteristic of the species rather than being associated with disbudding in cosmos. Casparian strips are frequently observed in the flowering stems and even in the non-flowering stems of this species, and also in chrysanthemum. They have not been observed in stems of either disbudded or flowering plants of petunia.

AUSTIN (2) concluded from his study of exflorated plants that "old age and death in the soy-bean are due to circumstances which accompany the reproductive phase but are not the direct result of it."

The preceding evidence from disbudded plants of cosmos and petunia seems to indicate that the differentiation of the structure of the flowering stem precedes or accompanies and is not a result of flowering. Thus the presence of a zone of thick-walled cells and the decrease of cambial activity of the flowering stem are not due to the production of flowers but to a physiological condition which is responsible for both.

### Summary

1. The flowering stems of all the species examined seem to have certain anatomical characteristics in common, regardless of age or of photoperiodic classification.

2. In contrast to the non-flowering stem, the flowering stem is characterized by: (1) a less active cambium; (2) a zone consisting mainly of thick-walled secondary xylem elements lying adjacent to the cambium in contrast to rather numerous vessels and thin-walled parenchymatous cells in the last formed xylem of the non-flowering stems; (3) generally thicker walls of the cells of the pericycle, perimedullary zone, xylem, and phloem; (4) freer staining with "basic" dyes of the pericycle, perimedullary zone, and certain elements of the xylem.

3. Certain specific differences in the flowering stems examined seem to be directly correlated with the "degree of reproductiveness" which the species attains.

4. Xylem elements which are characteristic of the flowering stem

are produced by plants of cosmos and *Ricinus* which were induced to produce a second set of flowering branches following a vegetative period.

5. The consistent differences in structure, which accompany the changes in character of growth as seen in the sections from stems of plants bearing aborting floral buds and of plants producing a second set of flowers following an interval of non-reproductive growth, are offered as evidence that the anatomy of the stem is related to the reproductive character of the plant.

6. The stems of disbudded plants have a structure resembling that of a flowering stem, which seems to indicate that the characteristic structure accompanies and is not a result of the production of flowers.

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# ZINC RELATION IN MOTTLE-LEAF OF CITRUS

A. R. C. HAAS

(WITH SEVEN FIGURES)

## Introduction

As a result of the work of CHANDLER, HOAGLAND, and HIBBARD (1) on little-leaf or rosette of plum trees, JOHNSTON (6) demonstrated that the application of zinc sulphate to the citrus tree or soil is usually followed by a marked improvement in the condition of the tree. Subsequently THOMASON (9), PARKER (8), and others extended greatly the treatments used by JOHNSTON.

This paper reports data obtained regarding several phases of the problem of the zinc relation in mottle-leaf of citrus: (1) effect of zinc treatment on the disappearance of mottling and on the rooting of mottled cuttings; (2) sugar content of leafy-twigs cuttings of Valencia orange in relation to mottling; (3) artificial production of mottle-leaf in soil and sand cultures; and (4) relation of zinc to the presence of mottle-leaf in the early stages of growth in solution cultures.

## Investigation

### EFFECT OF ZINC SULPHATE ON DISAPPEARANCE OF MOTTLING AND ON ROOTING OF MOTTLED CUTTINGS

These effects were studied by means of cuttings from healthy and from mottled Valencia orange trees grown at the University of California, Citrus Experiment Station. Healthy and mottled cuttings were used as untreated controls, while other healthy and mottled cuttings were dipped in the following mixture: 100 gm. of  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 50 gm. of  $\text{Ca}(\text{OH})_2$ , a minute amount of blood albumin spreader, and 1 liter of distilled water.

A similar experiment was also set up in which leaf cuttings of Valencia orange were used. These consisted of the whole blade and the petiole without axillary buds or portions of the twigs; hence no new top growth was possible, all growth necessarily having to take place in the root system.

The cuttings were planted in river gravel over a deep layer of crushed rock in bottom-heated (80° F.) rooting chambers. The cuttings were sprinkled with distilled water from a tinned sprinkling can in order to keep the additions of zinc to the untreated control cuttings at a minimum.

The two types of cuttings of Valencia orange were grown in the gravel beds from November 1, 1934 until February 16, 1935, during which time the new shoots of the leafy-twig cuttings were not permitted to develop. On February 16, 1935, the two types of cuttings were removed and the effect of zinc on their rooting was noted (table I). The greater fresh weight of the root systems produced by the zinc-treated mottled Valencia leaves may be due to the greater average fresh weight of these treated mottled leaves.

Although the average fresh weights of the mottled orange leaves are less than those of the healthy leaves, nevertheless the average fresh weights of the root systems of the mottled leaves are greater than those of corresponding healthy leaves.

The healthy Valencia leafy-twig cuttings were grown  $3\frac{1}{2}$  months in the rooting chamber. The adhering zinc-mixture residue was wiped from the leaves and the leaves of the control plants also were wiped. The zinc-treated healthy leaves had a deep blue-green color while the untreated healthy leaves had a yellowish or dull brownish green color. In the healthy Valencia leafy-twig cuttings used in these experiments there was a marked improvement in leaf color as a result of the zinc treatment.

The dense stand of zinc-treated mottled Valencia leafy-twig cuttings is shown in figure 1A, while figure 1B shows the sparsely leafed, untreated (control) mottled cuttings. In the period of the experiment not all of the mottling disappeared from the zinc-treated cuttings. The general color and the number of surviving leaves of the zinc-treated cuttings surpassed those of the untreated control cuttings.

The healthy Valencia leafy-twig cuttings (table I) showed the largest dry weights in the leaves and the smallest in the roots, while the mottled leafy-twig cuttings of orange showed the largest dry weights in the twigs and the smallest in the roots.

An advantage of the zinc treatment of mottled leafy-twig cuttings



FIG. 1.—Mottled leafy-twig cuttings of Valencia orange grown in rooting chamber: *A*, cuttings first dipped in zinc mixture (residues removed by wiping the leaves prior to taking photograph); *B*, cuttings without zinc treatment (leaves also wiped). Note dull color and thin stand of control cuttings.

TABLE I  
EFFECT OF ZINC ON ROOTING OF HEALTHY AND MOTTLED VALENCIA ORANGE AND  
LISBON LEMON LEAVES AND LEAFY-TWIG CUTTINGS

Cuttings	TREATMENT DIPPED IN ZINC MIXTURE OR NOT DIPPED (CONTROL)	TOTAL NO. OF CUT- TINGS	PERCENTAGE			AVERAGE FRESH WEIGHT PER ROOT SYSTEM (GM.)	AVERAGE FRESH WEIGHT PER TWIG (GM.)	AVERAGE DRY WEIGHT PER ROOT SYSTEM (GM.)	AVERAGE DRY WEIGHT PER TWIG (GM.)
			Rooted	Leaves (Dead)	Alive but not rooted				
Valencia orange									
Healthy leaves.....	{Dipped Not dipped	50 50	28 20	36 46	36 34	0.147 0.143			
Mottled leaves.....	{Dipped Not dipped	50 50	30 6	40 56	30 38	0.752 0.587			
Healthy leafy-twig.....	{Dipped Not dipped	50 195* 50 198*	98 100	0 4	2	0.877 0.949	0.663 0.600	0.135 0.137	0.271 0.244
Mottled leafy-twig.....	{Dipped Not dipped	50 228* 50 221*	100 82	1 20	18	0.629 0.617	0.594 0.578	0.170 0.166	0.230 0.245

\* Leaves on leafy-twig cuttings.

TABLE I—Continued

CUTTINGS	TREATMENT DIPPED IN ZINC MIXTURE OR NOT DIPPED (CONTROL)	TOTAL NO. OF CUT- TINGS	PERCENTAGE			AVERAGE FRESH WEIGHT PER LEAF (GM.)	AVERAGE FRESH WEIGHT PER ROOT SYSTEM (GM.)	AVERAGE FRESH WEIGHT PER TWIG (GM.)	AVERAGE DRY WEIGHT PER ROOT SYSTEM (GM.)	AVERAGE DRY WEIGHT PER TWIG (GM.)
			Rooted	Leaves (Dead)	Alive but Not Rooted					
Lisbon lemon										
Healthy leaves.....	{Dipped {Not dipped	54 54	52 26 67	48 67	7	1.43	0.464			
Mottled leaves.....	{Dipped {Not dipped	70 50	60 64	39 36	1	0.787	0.300			
Healthy leafy-twig.....	{Dipped {Not dipped	51 206* 53 218*	90 92	26 22	10 8	1.27 1.19	1.24 1.43	1.37 1.35		
Mottled leafy-twig.....	{Dipped {Not dipped	60 344* 51 282	100 100	6 3		0.66 0.63	0.988 1.46	1.12 1.26		



of orange is seen in the greater number that rooted and in the greater vigor of the leaves, as evidenced by the few leaves that died.

In table I, if the number of leaves on the Valencia twig cuttings that lived is multiplied by the average fresh weight per leaf, the following total fresh weights of the leaves are obtained: healthy dipped, 171.02 gm.; healthy control, 166.06 gm.; mottled dipped, 141.53 gm.; mottled control, 108.59 gm. These results suggest that the leaves of the mottled Valencia leafy-twig cuttings had a smaller fresh weight than the leaves of the healthy leafy-twig cuttings and yet produced the heavier root systems. The grams of fresh roots produced per gram of fresh leaves were as follows: healthy dipped, 0.272; healthy control, 0.277; mottled dipped, 0.403; mottled control, 0.369.

Lemon cuttings were also studied similarly. The leaf cuttings were grown from February 22 to April 17, 1935, at which time the mottling had disappeared from practically all of the leaves. The data obtained are presented in table I.

The zinc appears to have increased the rooting of healthy but not of the mottled leaf cuttings. The fresh weight of average root systems was 33 per cent of the fresh weight of an average healthy zinc-treated lemon leaf cutting and 38 per cent of the fresh weight of an average mottled zinc-treated leaf cutting. The relation of the fresh weight of roots produced to the weight of the zinc-treated leaf cuttings was therefore approximately the same for healthy and mottled leaves.

In table I are given also the data obtained in the rooting of lemon leafy-twig cuttings. A larger percentage of leaves of the healthy than of the mottled leafy-twig cuttings died. An average leaf of the mottled leafy-twig cuttings was only about half the fresh weight of an average leaf of the healthy leafy-twig cuttings of lemon. A slightly greater percentage of the leaves of treated than of untreated leafy-twig cuttings died. All of the mottled leafy-twig cuttings of lemon rooted while 90 to 92 per cent of the healthy ones rooted.

The grams of fresh roots per gram of fresh leaves of the leafy-twig cuttings were determined: healthy treated, 0.293; healthy control, 0.344; mottled treated, 0.278; mottled control, 0.430. These results (if the twigs are not taken into consideration) indicate a retardation

of root growth when the leaves of the healthy and mottled lemon leafy-twigs cuttings were zinc-treated, and show that the leaves of the control mottled cuttings were associated with a greater root production than were those of the control healthy cuttings.

The grams of fresh roots produced per gram of fresh twig (without leaves) of the leafy-twigs cuttings of lemon were: healthy treated, 0.905; healthy control, 1.061; mottled treated, 0.884; mottled control, 1.155. These results for twigs and roots bear the same relation as those for leaves and roots.

EFFECT OF ENVIRONMENTAL FACTORS.—During the course of these experiments the observation (3, p. 541) was repeatedly made, regarding the increase in green color of mottled leaves, that during the rooting process the mottled leaves in many cases became dark green and healthy in appearance, although the shape of the severely mottled leaves underwent no change. At least three factors may operate to bring about this recovery process: (1) the reduction in the intensity and the change in the quality of the light may affect the cuttings grown in the rooting chamber; (2) the degree of temperature (about 28° C.) and the relative humidity of the rooting chamber in conjunction with the reduced and altered light may favor vegetative growth; (3) the cuttings may absorb appreciable zinc from the river gravel in the rooting chamber.

Citrus trees when grown in the field under cloth or indoors under glass without direct sunlight produce luxuriant, tender, green, vegetative growth. The south-exposed portion of the trees in the field are often the most severely mottled. The duration of period of exposure to, and intensity of, sunlight therefore appear to be associated with mottle-leaf.

Severely mottled cuttings of orange and lemon recover partially or fully their green color when planted in gravel in rooting chambers regardless of whether the leaves are dipped in water, in a mixture of hydrated lime, in blood albumin spreader, or in this mixture plus zinc. Light, temperature, and humidity under these conditions are factors favoring a healthy appearance. In the field, leaves of the late autumn cycle that mottle as lower temperatures prevail may become fully green in the spring upon the return of higher temperatures.

The leaves of leafy-twigg cuttings of lemon grown in a culture solution to which no zinc was added, except as impurities, became yellow near the tip and tipburned when grown under continuous illumination. Some of the leaves showed a suggestion of mottle.

Various mottle patterns were found in the leaves when leafy-twigg cuttings of Valencia orange were grown in culture solutions in much used, 4-liter capacity, flint glass battery jars each containing three cuttings and with no zinc added except as impurities. The culture solutions were changed only at long intervals and the cultures were subjected to strong continuous illumination. Mottle also occurred under these conditions when 1 p.p.m. of zinc was added to the culture solution, but not when larger concentrations were used. Intense light no doubt can increase the requirements of a plant for a given element such as zinc. Mottle-leaf of citrus frequently is most severe in the bright hot interior districts of southern California.

In order to eliminate the possibility of the absorption of zinc and other materials from the river gravel as factors in the recovery from mottling while in the rooting chambers, experiments were conducted using pure Ottawa silica sand. Such sand was repeatedly acid-treated and washed with distilled water and placed in acid-washed glass or Swedish enamelware pans. Severely mottled leafy-twigg cuttings of lemon were placed in the sand. These sand cultures were then stored in the rooting chambers and within a few weeks the leaves showed a considerable increase in green color and the mottling became inconspicuous.

GERICKE (2) has shown that wheat plants grown in solution cultures devoid of iron remain green when grown in light of low intensity and produce excellent growth; whereas when the cultures are devoid of iron and are exposed to bright sunlight, the plants make little growth and become markedly etiolated. It is known that while light acts as a catalyst in chlorophyll formation, it also acts as a destroyer of chlorophyll, so that the net effect of these two processes becomes of importance. Light, temperature, and drainage effects may not be ignored as factors in studies of the effect of zinc on mottle-leaf, especially in cases of spontaneous recovery of trees from mottle-leaf when grown in soils.

In citrus the last cycle of growth which frequently matures during

the cold weather of autumn is the most likely to mottle. The effects of temperature on the solubility of soil constituents, on the absorption by the roots, and on photosynthesis, are of importance in the production of mottle-leaf and in the maintenance of an adequate supply of zinc and other constituents.

In walnuts it is usually the first cycle of growth that tends to mottle. Unless migration of zinc and other elements back into the twigs takes place, in any given season the abscission of the leaves in the late autumn may reduce the supply of zinc and other constituents available for the rapid development of the first cycle in the following spring. The rate at which traces of elements are available in relation to the rapidity of growth is an important consideration.

#### SUGAR CONTENT OF LEAFY-TWIG CUTTINGS OF VALENCIA ORANGE IN RELATION TO MOTTLING

The environmental conditions in the rooting chamber were similar for all the cuttings. No new top growth was allowed to develop. While in effect this may be considered as a form of pruning, it was carried on similarly in all cuttings. Only the growth that took place in the roots was allowed to remain.

At the end of the experiments the various portions of the different lots of leafy-twig cuttings were rapidly washed with distilled water, dried between filter paper and wiped with diaper cloth. The plant fractions were then rapidly dried below 65° C., after which they were ground in a Wiley mill.

The data for the sugar determinations (table II) suggest that the zinc treatment lowered the percentages of reducing and total sugars. A large percentage of total sugars, chiefly sucrose, was found in the roots. The sugar content of healthy and of mottled citrus leaves was further investigated because it has been shown that mottled leaves are richer in nitrogen, phosphorus, and potassium but lower in calcium than healthy leaves; and because frequently in the field it is observed that, unless in too advanced a stage of mottling, mottled trees (for a given size of the top) may show no appreciable decreased fruit production below that of healthy trees of the same size. It is not known how severe the mottle-leaf condition must be in order to affect the yield.

Further data were obtained regarding the sugar content of healthy and of mottled citrus leaves. The samples were collected sometimes from single trees or sometimes from numerous trees. In each case the healthy and mottled samples were obtained from the same trees at approximately the same time. This procedure was followed because rarely are healthy trees found that do not bear some mottled leaves.

TABLE II  
PERCENTAGE REDUCING AND TOTAL (AS REDUCING) SUGARS IN DRY  
MATTER OF LEAVES, TWIGS, AND ROOTS OF VALENCIA  
ORANGE LEAFY-TWIG CUTTINGS

HEALTHY CUTTINGS				MOTTLED CUTTINGS			
DIPPED IN ZINC MIXTURE		NO ZINC (CONTROL)		DIPPED IN ZINC MIXTURE		NO ZINC (CONTROL)	
REDUCING SUGARS	TOTAL AS REDUCING SUGARS	REDUCING SUGARS	TOTAL AS REDUCING SUGARS	REDUCING SUGARS	TOTAL AS REDUCING SUGARS	REDUCING SUGARS	TOTAL AS REDUCING SUGARS
Leaves 1.62.....	2.88	1.70	3.18	1.74	3.58	1.86	4.77
Twigs 1.32.....	3.05	1.78	3.17	1.45	3.72	1.80	3.73
Roots 1.49.....	6.35	1.47 ✓	6.51	1.71	6.63	2.75	8.08

The data for the reducing and the total (as reducing) sugars are given in table III. The dry matter of mottled leaves contains slightly greater percentages of reducing and total sugars than that of healthy leaves; such higher content may well account for increases in the percentage of rooting, for the total amount of roots produced in cuttings, and for the apparently quicker response of mottled over that of healthy trees to applications of zinc in the field.

#### ARTIFICIAL PRODUCTION OF MOTTLE-LEAF IN SOIL AND SAND CULTURES

It was difficult to produce mottle-leaf out of doors when the trees were grown in sand cultures in large galvanized iron containers.

This may have been due to penetration by the root of the asphalt paint on the inside of the container whereby the trees obtained a supply of zinc. The closest approach to mottle-leaf as it occurs in the field was produced in well drained sand or soil cultures in asphalt-coated zinc cans, in earthenware pots, or in wooden containers when

TABLE III  
PERCENTAGE REDUCING AND TOTAL (AS REDUCING) SUGARS  
IN DRY MATTER OF CITRUS LEAVES

VARIETY	COLLECTED	LOCATION	REDUCING SUGARS		TOTAL AS REDUCING SUGARS	
			LEAVES NOT MOTTLED	LEAVES MOTTLED	LEAVES NOT MOTTLED	LEAVES MOTTLED
Navel orange on sour orange stock.....	Dec. 11, 1930	Pathology plots, R6, T5, 6, 7	2.20	2.58	4.14	4.40
Marsh grapefruit on sweet orange stock.....	Dec. 11, 1930	Pathology plots, R18, T3	2.29	2.74	3.74	5.60
Valencia orange on sweet orange stock.....	Jan. 9, 1933	Plot A, Rubidoux	1.95	2.10	2.96	3.04
	Jan. 12, 1933	Plot G, Rubidoux	1.80	2.09	4.46	4.20
	Jan. 12, 1933	Plot H, Rubidoux	1.75	2.13	5.90	5.90
	Feb. 3, 1933	Plot K, Rubidoux	1.95	2.74	5.76	6.42
	Feb. 17, 1933	Plot S, Rubidoux	1.82	2.19	8.14	7.48
	Feb. 18, 1933	Plot V, Rubidoux	2.17	2.32	3.56	3.66

the cultures received a solution containing urea, arsenic, cyanamid, or dicyanamid (figs. 2, 3). Calcium nitrate was usually added in conjunction with these materials, especially when they were used in high concentrations. Calcium nitrate alone failed to produce the symptoms in the leaf. When the substances were used without calcium nitrate the injury was often sudden and severe. The addition

of calcium nitrate usually was accompanied by an increase in growth which lessened the injury and tended to show more of the intermediate stages.

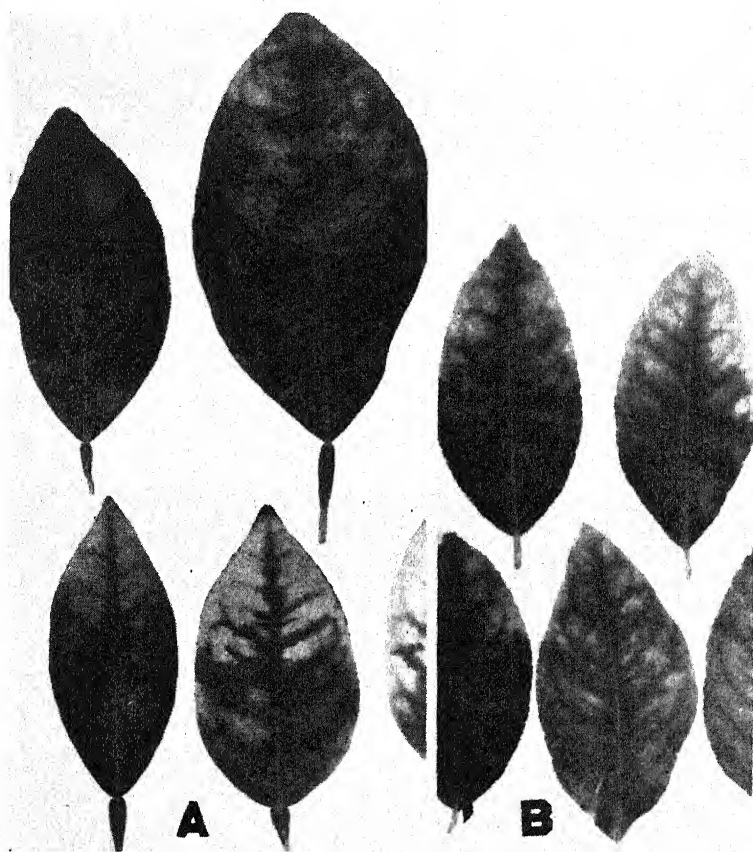


FIG. 2.—Type of mottle in leaves from trees grown in either Sierra loam soil or sand cultures in galvanized iron containers, glazed crocks, or boxes, with addition of urea-calcium nitrate mixture to the soil or sand: A, Valencia orange (upper left, leaf from control culture); B, lemon.

Some of the symptoms of mottling produced in artificial cultures may not be considered by some investigators as being the "true" mottle-leaf as seen in the field, although there are many different so-called types of mottling that extend over a rather wide range of

patterns. A mottled condition exists when the chlorosis does not involve the veins. One criterion as to whether a chlorosis is a true mottle is the degree of recovery brought about by the use of zinc.

In the artificial production of mottle-leaf, difficulty was encountered in supplying sufficient iron to prevent complete chlorosis because of the tendency toward alkalinity and in preventing in the presence of considerable calcium in the urea mixture the appearance of symptoms of potassium and boron deficiency. The use of urea or

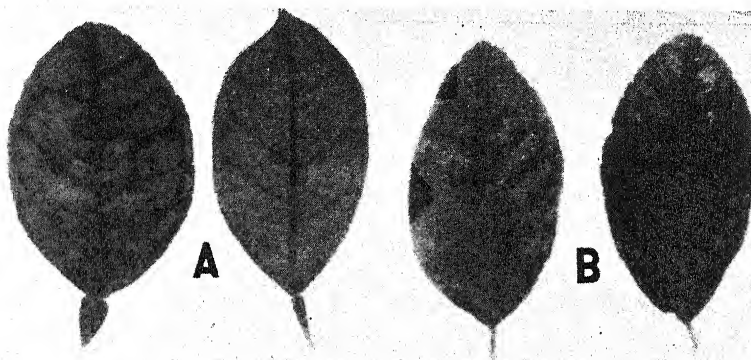


FIG. 3.—Various types of mottle produced in citrus leaves: *A*, left, grapefruit leaf from leafy-twig cuttings grown in solution or in Sierra loam soil cultures containing excessive arsenic; right, Valencia orange leaf from trees grown in pot cultures with soil containing excessive arsenic in any of several forms. *B*, effect of excessive cyanamid or dicyanamid on lemon leaves from budded trees in soil cultures in galvanized iron containers.

a urea-calcium nitrate mixture, cyanamid, dicyanamid, or arsenic was accompanied by varying degrees of partial chlorosis, depending on the amount and frequency of adding the materials to the soil. Large amounts, particularly of the urea-calcium nitrate mixture, brought about severe symptoms, including little-leaf. By regulating the amounts applied, the frequency of their addition, and the extent of drainage, the appearance of symptoms of chlorosis in the new growth could be controlled. As the nitrification of the urea to nitrate progresses, the new leaves produced are healthy and the affected leaves show some improvement. Similarly in soil cultures in wooden containers or in galvanized iron tanks as large as 8 feet in diameter and 4 feet deep, typical little-leaf and a mottled type of



chlorosis could be brought about, not only in citrus but also in walnuts (fig. 4), by controlling the application of urea-calcium nitrate mixture.

Calcium nitrate alone showed no injurious effect. The severity of the disease could be controlled as previously described for citrus.

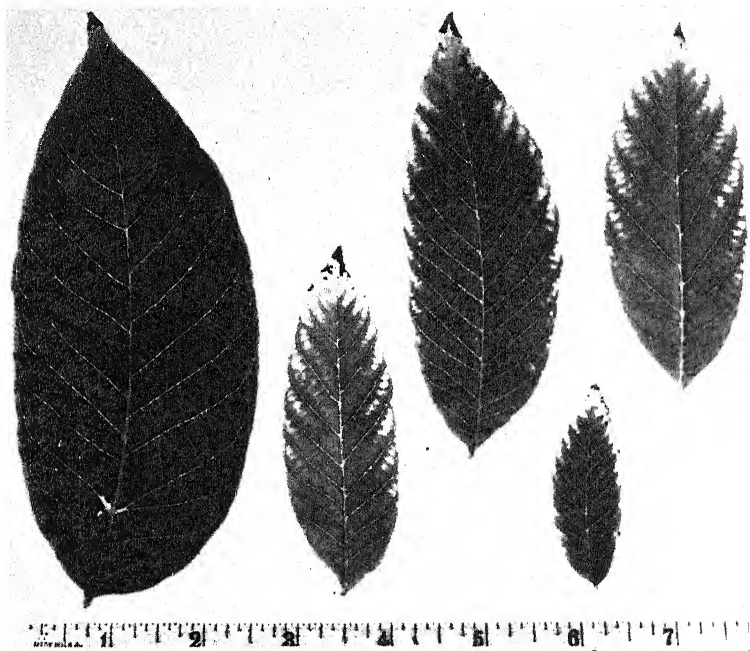


FIG. 4.—Walnut leaflets from trees grown in Sierra loam soil in galvanized iron tanks: Left, control; remaining leaflets show varying degrees of chlorosis between the veins. Symptoms resulted from use of various urea-calcium nitrate mixtures or urea alone but not calcium nitrate alone. Reduction in size of leaflets and severity of mottle could be controlled by the time and amount of application.

There is one point of similarity between the artificial production of the type of mottle in walnut leaves in soil cultures and that which occurs in the field, in that under artificial conditions the mottle was always produced on the first cycles of growth but never on the late cycles. In the field it is the first cycle of growth that is by far the most severely affected; in many cases the later cycles of growth appear healthy.

Since urea and many of the other compounds employed contain the  $\text{NH}_2$  group, it is possible that a mottle type of chlorosis may be associated with the nitrogen group when the absorption of large amounts of such incompletely nitrified constituents takes place. Zinc is an element known especially for its oxidation-reduction activity and for its catalytic functions. The zinc absorbed from the soil and container appears to be adequate when only small amounts of the urea mixture are applied, but with large amounts the zinc absorption possibly cannot keep pace with the absorption of these incompletely nitrified constituents. Further studies of the relation of absorbed to assimilated nitrogen groups are to be undertaken.

The use of  $\text{NaNO}_3$  in soil cultures without drainage was associated with increasing alkalinity and mottling in citrus, and especially in pecans. The alkalinity may have greatly reduced the concentration of soluble zinc and other elements essential for healthy growth; while the excessive nitrate, before injury was manifest, further reduced the relative amounts of these constituents in the tissues by the dilution factor brought about by the stimulation of active vegetative growth.

When the leaves of orange trees grown in cultures containing urea were dipped in a zinc-lime-spreader mixture the mottle became less pronounced, although the time allowed for the zinc to act also gave the nitrification processes in the soil more time in which to transform the urea more completely to the nitrate stage, so that the tests lacked adequate control.

VAILE (10) has pointed out that the use of nitrogen in the form of nitrate of soda and to a less degree in the form of dried blood, without the use of bulky organic manure of some sort, is conducive to the development of mottle-leaf in citrus trees. Leaves of trees on plots without nitrogen additions showed practically no mottling. These deductions may have been based on the fact that trees which receive no nitrogen make comparatively little new growth, and the requirement under such conditions for zinc and other constituents is extremely small compared with the requirements when relatively pure nitrogen fertilizer additions have induced marked vegetative growth. Bulky organic manures on the other hand contain fairly large amounts of a considerable number of constituents other than nitrogen, among which is zinc.

RELATION OF ZINC TO PRESENCE OF MOTTLE-LEAF IN EARLY  
STAGES OF GROWTH IN SOLUTION CULTURES

It was shown by HAAS (3) with lemon cultures that zinc in minute amounts in the culture solution was of benefit to the quality of the growth, while somewhat greater amounts were distinctly injurious.

Some of the factors that have retarded the drawing of conclusions on the effect of zinc on citrus are the minute amounts necessary for growth, the difficulty of preventing zinc contamination, the need of chemical methods for determining minute amounts (5), and the absence of an adequate procedure for growing citrus in solution cultures.

Until recently, leafy-twigg cuttings of orange could not be grown in culture solutions on their own roots beyond a certain stage. Considerable success, however, was had in growing such cuttings when they were used as scions on lemon or rough lemon leafy-twigg cuttings as stocks, and the more so as the phosphorus nutrition in relation to that of the minor elements was better understood.

In order better to determine the causal factors involved in mottle-leaf, it is essential that mottling be produced in solution cultures. Although no zinc was added to solution cultures (except as impurities in the so-called "chemically pure" salts), mottling thus far has not been produced with lemon or rough lemon leafy-twigg cuttings alone, or with leafy-twigg cuttings of orange as scions on lemon or rough lemon leafy-twigg cuttings as stocks. This emphasizes the importance of the results of LEE (7), who found that a given variety may differ in its susceptibility to mottle according to the stock on which it is grown.

In order to test the effectiveness of zinc upon growth and upon the incidence of mottle-leaf, unaerated solution cultures were conducted with rooted leafy-twigg cuttings of Valencia orange in Swedish enamelware shallow pans. The cuttings were placed in the cultures on October 19, 1934 and were photographed on April 25, 1935. The culture solution employed (4) was that previously used by HOAGLAND, and contained the single strength calcium nitrate with iron, boron, and manganese added as minor elements. Phosphate was present in the solution for only a few days at intervals of several weeks. When it was absent from the solution, then aluminum or

zinc or both were added. The pH values of the culture solutions were raised to approximately 6 by means of a solution of calcium hydroxide.

Healthy growth was obtained when either 0.5 p.p.m. zinc or 0.5 p.p.m. aluminum or both were present in the culture solution. Increased growth, however, was obtained when 1 p.p.m. zinc or 1 p.p.m. aluminum or both were present. These approximate concentrations furnish a basis for subsequent studies relative to zinc in culture solution.

At higher concentrations of zinc, as HAAS (3) has previously pointed out, injurious symptoms of zinc poisoning occur. In figure 5*B* it is seen that when 5 p.p.m. of zinc were present the smallest amount of growth resulted. The roots at first appeared healthy but soon showed injury. The use of 5 p.p.m. aluminum was accompanied by excellent growth when zinc was not added. Addition of aluminum resulted in improved growth when the culture solution contained an excess of zinc.

Control cultures were conducted in which neither zinc nor aluminum was added to the solution. Figure 5*A*, left, shows the growth made by such cultures. The leaves were quite yellow. Figure 5*A*, right, shows another such culture but which received 5 p.p.m. aluminum several months after the culture was started. The root growth was increased and the leaves were dark green.

In every case the use of zinc brought about a dark green color in the leaves without the appearance of mottling. It is of interest that when aluminum was used without zinc (fig. 5*A*), mottling occurred even though the leaves of the original cuttings were dark green and healthy in appearance (fig. 6*A*). It should be emphasized that this is the first instance of the artificial production of mottle-leaf in controlled solution cultures and forms a basis for further investigations on the zinc relation in mottle-leaf. The omission of zinc from the culture solution was not accompanied in every case by mottling, and it remains for future studies with more highly purified solutions to ascertain the reasons.

Badly mottled leafy-twig cuttings of Valencia orange are shown in figure 6*B*. The solution contained 5 p.p.m. of aluminum but no added zinc. The mottle disappeared when zinc was added.

In order to study the effect of coating leaves with zinc mixture, rooted leafy-twig cuttings of Valencia orange were grown in a culture solution to which no zinc was added. Some of the tops were dipped

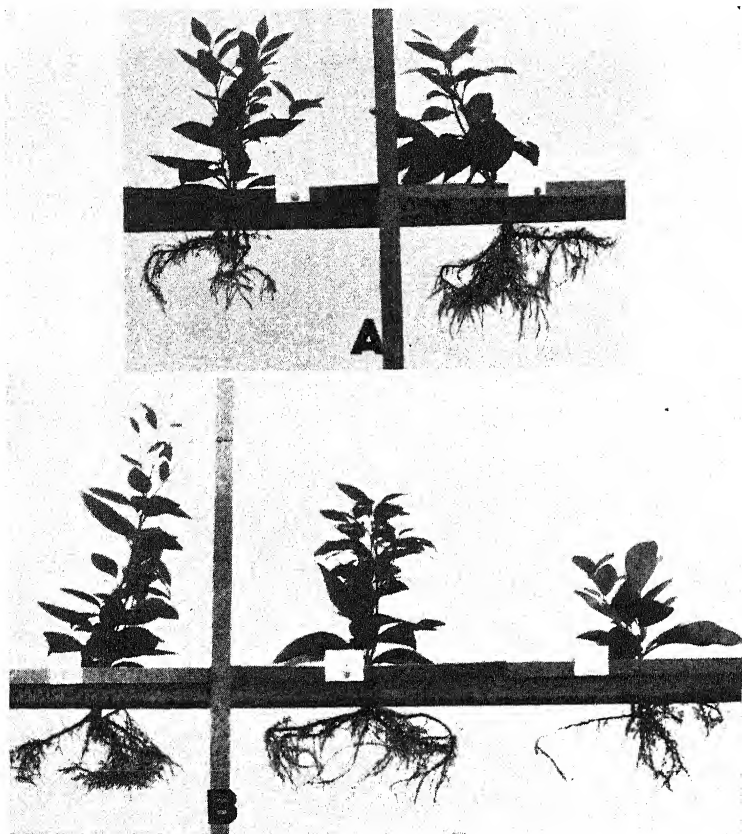


FIG. 5.—Leafy-twig cuttings of Valencia orange grown in culture solution: *A*, left, without zinc or aluminum added; right, without zinc but with 5 p.p.m. aluminum added several months after culture was started. *B*, left, 5 p.p.m. aluminum; right, 5 p.p.m. zinc; center, 5 p.p.m. zinc and 5 p.p.m. aluminum.

in the mixture, while the leaves of some cuttings were coated on the upper side and those of others were coated on the lower side.

The tops of cultures (fig. 7*A*) were dipped in the zinc mixture while the leaves of others (fig. 7*B*) were painted with the mixture

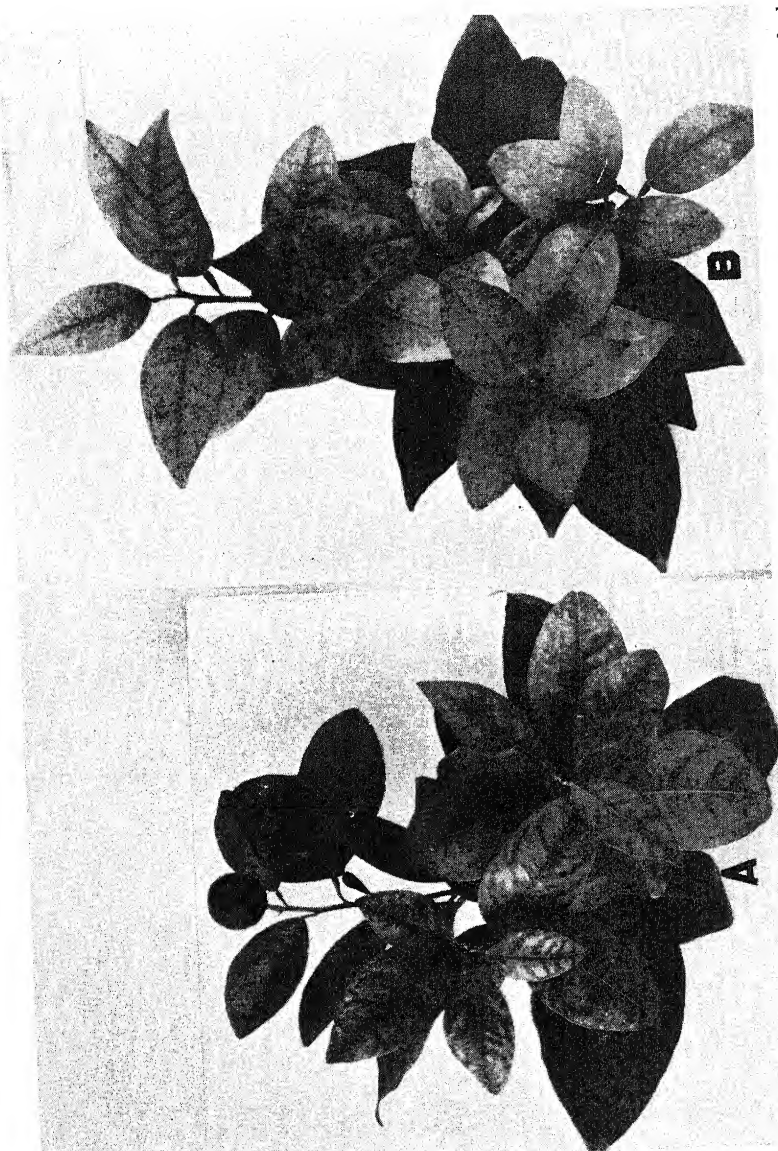


Fig. 6.—Severe mottling of Valencia orange leafy-twig cuttings in culture solution: *A*, same as figure 5*A* but photographed to show mottling of new leaves. Note dark green, healthy leaves of original cuttings. *B*, 5 p.p.m. aluminum, no zinc; recovery followed addition of zinc.

on the upper surface only. The cultures were grown from December 28, 1934 to April 25, 1935. At the end of this period the cuttings of which the leaves were coated only on the upper surface made the greatest initial growth. When the zinc coating was applied to both the upper and lower surfaces, growth was retarded.

Subsequently these cuttings made healthy growth, surpassing in quantity and quality that of cuttings not apparently checked. Earlier in this paper it was shown that the zinc treatment retarded the root growth of a lot of lemon leafy-twigg cuttings. Such temporary or transient toxic effects of zinc absorption, as well as the state of

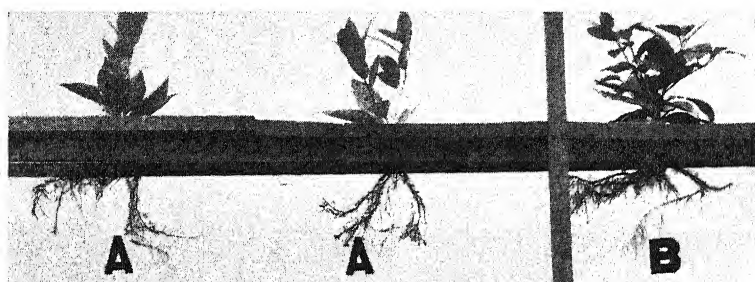


FIG. 7.—Effect on growth of coating surfaces of leaves of solution cultures of Valencia orange leafy-twigg cuttings with mixture of zinc. A, leaves dipped in mixture; B, only upper surface of leaves coated.

the nutrient reserves in the tree, possibly may account in part for the marked variations that frequently are noted in the response of trees to zinc sprays.

### Summary

1. With both healthy and mottled Valencia leaves the zinc treatment increased the number of leaves that rooted. Twenty-eight per cent of the zinc-treated healthy Valencia leaves and 20 per cent of the control (not treated) healthy leaves rooted. Thirty per cent of the zinc-treated mottled Valencia leaves and 6 per cent of the control (not treated) mottled leaves rooted. Fewer leaves died among the zinc-treated than among the control leaves.

2. Although the average fresh weights of the mottled orange leaves are less than those of the healthy leaves, the average fresh

weights of the root systems of the mottled leaves are greater than those of the corresponding healthy leaves.

3. Of the healthy lemon leaf cuttings dipped in zinc mixture, 52 per cent rooted; of those not dipped (control), only 26 per cent rooted. Zinc treatment benefited the rooting of healthy leaves but failed to improve the rooting of mottled lemon leaves. Over 60 per cent of mottled lemon leaf cuttings rooted regardless of whether zinc treated or not. Mottled lemon leaf cuttings root as readily if not better than healthy lemon leaves.

4. The leaves of mottled Valencia leafy-twigg cuttings had a smaller fresh weight than the leaves of healthy leafy-twigg cuttings, and yet produced the heavier root systems. The treatment of mottled Valencia leafy-twigg cuttings with zinc mixture increased the grams of fresh roots per gram of fresh leaves.

5. The zinc treatment retarded the root growth of lemon leafy-twigg cuttings. The untreated mottled lemon leafy-twigg cuttings produced a greater fresh weight of roots than the untreated healthy lemon leafy-twigg cuttings.

6. The various portions of untreated (control) rooted mottled Valencia leafy-twigg cuttings contained greater percentages of sucrose than the corresponding portions of healthy (not mottled) cuttings. The zinc treatment lowered the percentages of sucrose present in the leaves and roots at the end of the experiment.

7. The dry matter of mottled leaves grown in the field contains slightly greater percentages of reducing and total sugars than does that of healthy leaves. This increased sugar content is of advantage in bringing about a rapid recovery once the limiting condition has been corrected.

8. In sand or soil cultures, an excess of urea, urea-calcium nitrate mixtures, cyanamid, dicyanamid, or arsenic brought about various stages of partial chlorosis. In the case of urea, little-leaf was induced, the severity of which could be controlled by regulating the amount and frequency of the applications.

9. Rooted cuttings grow well in solution cultures when phosphate is supplied for a few days at intervals of several weeks and when aluminum is present during the absence of phosphate.

10. Mottle-leaf was produced for the first time in Valencia leaves



of rooted leafy-twigg cuttings grown in culture solutions. In solution cultures mottling has been produced thus far only in the absence of zinc or in the presence of low zinc concentrations but under continuous high light intensities.

11. When zinc concentrations in the culture solution are somewhat excessive, the addition of aluminum gave beneficial effects on growth. The use of zinc in the culture solution in not too high a concentration was followed by a dark green color in the leaves.

12. By means of coating with a mixture of zinc the surfaces of leaves of rooted Valencia leafy-twigg cuttings grown in culture solutions, it was possible to note the toxic effect of zinc or its temporary inhibiting or retarding effect on growth. Such effects may partially account for the variations that frequently are observed in the rate of response of trees to zinc applications.

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EFFECT OF ATMOSPHERIC HUMIDITY ON RATE  
OF CARBON FIXATION BY PLANTS<sup>1</sup>  
CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 473

JOHN W. MITCHELL

(WITH FOUR FIGURES)

Introduction

That the synthesis of solid materials by many kinds of plants is inhibited when the surrounding air is relatively dry has been shown by several investigators (5, 10, 15, 18). LEBENDINCEV (12) and others have substantiated the view that a decrease in the water content of leaves is generally accompanied by a decrease in the rate of carbon fixation (2, 3, 4, 8).

The purpose of the present investigation was to determine whether any direct correlation between atmospheric moisture, rate of photosynthesis, and accumulation of dry weight by certain plants could be established. Leaves and also the entire tops of plants inclosed in glass chambers were supplied with air streams containing different known amounts of moisture, and the rate of carbon fixation measured. All environmental factors were controlled and maintained constant with the exception of those under consideration. The stomatal behavior and to some extent the quantitative carbohydrate content of leaves grown at different humidities were determined.

Methods

Plants were grown in a greenhouse in fertile soil contained in clay pots. They were placed in a room in which environmental factors were controlled, and supplied with artificial light during the measurement of carbon fixation rates (17). The soil was kept continually moist by placing the pots in shallow dishes that contained water about 1 cm. deep.

Methods and apparatus used in estimating the rate of carbon fixation by leaves have been described (17). These methods, how-

<sup>1</sup> This investigation was aided in part by a grant to the University of Chicago from the Rockefeller Foundation.

ever, were modified for estimating the rate of carbon fixation of the above-ground portion of plants. This modification consisted of encircling the stem of a plant with the two halves of a circular metal plate which had an opening in the center for the stem (fig. 1). The two metal pieces were held together with screws, the union sealed with a mixture of clay and castor oil, and the space between the

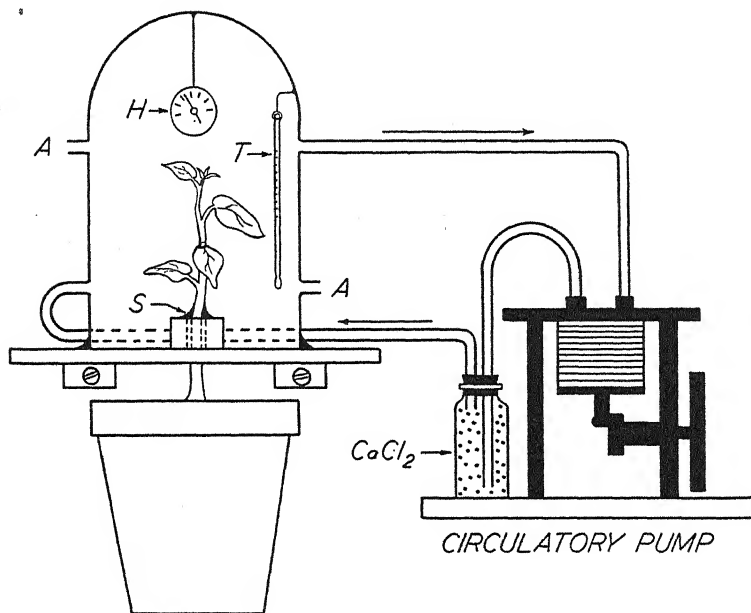


FIG. 1.—Apparatus used to study effect of relative humidity on rates of carbon fixation by plants: A, A, inlet and outlet for air stream; T, thermometer; H, hygrometer; S, clay seal.

stem and metal also sealed in the same manner. A glass chamber with suitable outlets was then placed over the part of the plant extending above the plate, and an airtight union was made between the glass and metal by means of the oil and clay mixture. A circulatory pump, attached by means of rubber tubing to outlets in the chamber, recirculated the inclosed gas through the pump and chamber at a rate of 130–180 liters per hour. By means of other outlets in the chamber (A, A), air slightly enriched with  $\text{CO}_2$  was passed through at a rate of 15.6 liters per hour, and the absorption of  $\text{CO}_2$

by the plant was determined by means of an electrical conductivity method (17).

The humidity of the air inclosed by this type of chamber was controlled by the use of a drying agent through which the air was circulated (fig. 1). Moisture was removed from air surrounding the inclosed leaves in the same manner. The relative humidity of the atmosphere was indicated by hygrometers which were suspended inside the chambers directly above the inclosed plants (*H*). These instruments were calibrated by suspending them in a bottle above solutions of sulphuric acid and water of various known concentrations. During the calibration, the bottle containing the hygrometer

TABLE I  
CALIBRATION OF HYGROMETER BY MEANS OF KNOWN HUMIDITIES PRODUCED OVER SOLUTIONS OF SULPHURIC ACID

PERCENTAGE OF ACID	TEMPERATURE (C°.)	SPECIFIC GRAVITY	RELATIVE HUMIDITY	READING
17.01.....	20.0	1.12	92.3	90.0
41.60.....	20.0	1.32	57.3	54.0
61.0.....	20.0	1.51	17.0	18.0
79.0.....	20.0	1.72	2.7	0.0

and acid was closed with a stopper through which were inserted a thermometer and inlet and outlet tubes to which a circulatory pump was connected and used to circulate the inclosed air. Various known humidities were produced in this manner (table I) and curves were plotted by means of which the hygrometer readings were corrected.

In determining the effect of atmospheric moisture on carbon fixation rates, the inclosed leaves or tops of plants were first exposed to moist air, then to relatively dry air, maintaining other environmental factors constant while the rate of carbon fixation was measured. Granular  $\text{CaCl}_2$  proved most suitable for removing moisture from the air. In some cases, however, solutions of sulphuric acid diluted with different amounts of water were used as drying agents with no apparent injury to the plants.

The method of carbohydrate analyses referred to later consisted in digesting the dried material in 2.5 per cent HCl for two and one half hours under a reflux condenser. The solution was cooled and

made to volume. It was then filtered and the filtrate neutralized with anhydrous sodium carbonate. Reducing sugars were then determined (19) and calculated as glucose.

### Experimental results and discussion

Recent papers (6, 10, 11) show that the rates of carbon fixation by many kinds of plants fluctuate during a given period of illumination under natural conditions. It was desirable to determine whether similar changes occurred in the rates of carbon fixation by plants grown in the constant environmental conditions used in these experiments. The rates at which carbon was fixed by attached leaves of *Lycopersicum esculentum* (Mill.), *Pelargonium zonale*, *Senecio cruentus* (DC), *Phaseolus vulgaris* (Linn.), and *Primula obconica* (Hance) under a constant environment were determined hourly over periods of 3-8 hours for intervals varying from two to fourteen days. The intensity of artificial light to which the leaves were exposed was constant for any individual experiment, but varied between 900 and 1200 foot candles between different experiments as measured with a Weston light meter. The temperature, humidity, and rate of circulation of air around the leaves were maintained constant, while variations in the concentration of carbon dioxide in air surrounding the leaves was maintained within the range of fluctuation found in the concentrations of CO<sub>2</sub> of air under natural conditions. The amounts of carbon fixed by the leaves of the plants mentioned varied slightly from hour to hour, during a given period of illumination. Data obtained from experiments with tomato leaves showed that the maximum deviation from the mean rate of carbon fixation exhibited by an individual leaf over a period of seven hours was approximately 14 per cent (table II). The hourly variations in the rates of carbon fixation of the leaves of other plants was generally less and seldom greater than this amount.

### EFFECT OF HUMIDITY ON RATE OF CARBON FIXATION

Six plants of *Primula obconica* were selected from a number grown in a greenhouse during the fall and winter and placed under artificially controlled conditions. One leaf on each plant was chosen with respect to size, position on plant, and color, and after 25 hours

these attached leaves were inclosed in individual chambers. Air at a temperature of 24.5–25.5° C. was enriched with CO<sub>2</sub> such that on leaving the chambers it contained this gas in amounts within the range of fluctuation found under natural conditions. The enriched air was drawn at the same rate through each chamber for two hours. During this time the moisture content of air surrounding the inclosed leaves increased to 70–80 per cent of saturation, due principally to transpiration. The amounts of CO<sub>2</sub> absorbed by the individual leaves when surrounded by air of this moisture content were meas-

TABLE II

CARBON DIOXIDE ABSORBED BY TOMATO LEAVES UNDER CONSTANT CONDITIONS. RESULTS CALCULATED AS MILLIGRAMS PER HOUR PER HUNDRED SQUARE CENTIMETERS OF LEAF SURFACE

No. OF LEAF	Hour						
	1ST	2ND	3RD	4TH	5TH	6TH	7TH
9.....	17.9	14.4	17.5	17.3	14.5	18.0	17.4
11.....	19.0	20.1	19.4	20.0	20.6	17.8	20.7
13.....	16.1	17.9	17.1	19.6	16.0	17.2	19.3
14.....	16.7	17.9	17.5	18.8	16.0	17.2	17.9
15.....	16.1	17.9	17.1	16.6	16.1	16.3	18.9
16.....	17.0	15.2	17.6	16.2	16.2	16.4	17.2
Average.....	17.1	17.2	17.7	18.1	16.5	17.2	18.6

ured during the third hour (table III). Flasks containing 20 per cent sulphuric acid were then connected between the circulatory pumps and plant chambers as previously described. The chambers were again flushed for two hours with enriched air, during which time the moisture content of the inclosed air decreased to 40–50 per cent of saturation. The amounts of CO<sub>2</sub> absorbed by the inclosed leaves at this humidity were determined during the third hour. The humidity of air in the chambers was reduced still farther by the use of 50 per cent solutions of sulphuric acid, and the procedure of flushing repeated. The amount of CO<sub>2</sub> absorbed by the inclosed leaves at a humidity of 10–20 per cent was determined. The leaves appeared turgid during exposure to low humidities and showed no evidence of wilting, although careful examinations were made immediately after

they were removed from the chambers. The plants were returned to the greenhouse. No evidence of injury occurred during the period of experimentation nor during the following three weeks.

The preceding experiment was repeated using attached leaves of bean, tomato, *Pelargonium*, *Brassica oleracea* (Linn.), and *Cucurbita pepo*. Dry  $\text{CaCl}_2$  and solutions of sulphuric acid were used to remove moisture from the air in the chambers. Results obtained with the leaves of these plants were similar to those obtained with the leaves

TABLE III  
CARBON DIOXIDE ABSORBED BY LEAVES OF PRIMULA  
WHEN SUBJECTED TO DIFFERENT HUMIDITIES. RE-  
SULTS CALCULATED AS MILLIGRAMS PER HOUR PER  
HUNDRED SQUARE CENTIMETERS OF LEAF SURFACE

No. OF LEAF	PERCENTAGE HUMIDITY		
	70-80	40-50	10-20
38.....	14.0	10.9	11.9
39.....	10.8	10.0	11.3
40.....	16.0	15.0	12.6
41.....	12.1	14.0	13.0
42.....	12.4	12.0	12.3
43.....	16.7	17.0	16.0
Average...	13.7	13.2	12.9

of *Primula* and in no case did the inclosed leaves wilt, exhibit injury, or show a significant reduction in the rate of carbon fixation when exposed for three-hour intervals to low humidities.

#### EFFECT OF WILTING ON RATE OF CARBON FIXATION

Six plants of *Cineraria* were selected from a number which had grown in a greenhouse during the fall and winter. One leaf attached to each plant was chosen with respect to size, position on stem, and color, in order to have them as nearly uniform as possible. The plants were placed under controlled environmental conditions and after 24 hours the selected leaves were inclosed in leaf chambers. Enriched air at  $24.5\text{--}25.5^\circ\text{C}$ . was drawn through the chambers and the amount of  $\text{CO}_2$  absorbed by the inclosed leaves determined during the third hour, as in previous experiments. No significant differ-

ences were noted in the rates of carbon fixation when the leaves were surrounded for three hours with air having a relative humidity of 70-80, 50-60, or 20-30 per cent (table IV). All the leaves remained turgid during the experiment and showed no evidence of injury after several weeks.

This experiment was repeated using older leaves of *Cineraria* taken from another group of plants and somewhat different results were obtained. Six leaves attached to individual plants were inclosed in leaf chambers after the plants had been under controlled environ-

TABLE IV  
CARBON DIOXIDE ABSORBED BY LEAVES OF CINERARIA  
WHEN SUBJECTED TO DIFFERENT HUMIDITIES. RE-  
SULTS CALCULATED AS MILLIGRAMS PER HOUR PER  
HUNDRED SQUARE CENTIMETERS OF LEAF SURFACE

NO. OF LEAF	PERCENTAGE HUMIDITY		
	70-80	40-50	10-20
1.....	14.9	15.1	16.2
4.....	11.0	9.0	12.0
6.....	13.8	13.8	13.0
18.....	16.0	14.6	16.1
21.....	14.2	13.8	13.3
23.....	13.1	12.9	15.0
Average...	13.9	13.2	14.3

mental conditions for 24 hours. The chambers were flushed with enriched air as previously described, and the amount of CO<sub>2</sub> absorbed by the leaves in moist air was measured during the following three hours. The moisture content of the air in the chambers was then reduced by means of CaCl<sub>2</sub> and the amount of CO<sub>2</sub> absorbed by the leaves when surrounded by relatively dry air was measured during the following three hours. Three of the inclosed leaves became slightly wilted when surrounded with dry air, and these showed a decrease of 30-40 per cent in the rate of carbon fixation, while the other three remained turgid and absorbed approximately the same amount of CO<sub>2</sub> whether in dry or in moist air. Later the plants were placed in a greenhouse at a temperature of 55° F., and all of the



wilted leaves regained turgidity within two hours and showed no apparent signs during several weeks following the experiment of having been injured.

It is evident from the preceding experiments that individual leaves of squash, bean, cabbage, *Pelargonium*, and tomato plants remained turgid at a temperature of 25° C. although subjected to extremely low humidities. *Cineraria* leaves, which are especially sensitive to dry atmospheric conditions, in some few cases lost turgidity when the humidity of the surrounding air was rapidly decreased, and the loss of turgidity was accompanied by a marked decrease in the rate of carbon fixation. Further experiments concerning the effect of wilting on the rate of carbon fixation were not conducted, as other investigators (2, 3, 4) have shown that the desiccation of plants is often accompanied by a decreased rate of carbon fixation. It is emphasized, however, that low atmospheric humidity did not cause wilting in most of the plants studied. It is evident that in some cases dry air induces wilting only when accompanied by some other environmental condition, such as high temperature or low soil moisture, which also tend to reduce the water content of the plant.

#### EFFECT OF INCREASED TEMPERATURE AND PROLONGED EXPOSURE TO LOW HUMIDITY ON RATES OF CARBON FIXATION

The leaves of *Pelargonium hortorum* were used in this experiment. The plants were 8-10 inches high and possessed about 15-25 leaves per plant. Six plants were selected from a group of several hundred and placed under the artificially controlled conditions previously described. The tops of the plants were inclosed in glass chambers (fig. 1) and the systems flushed with enriched air. The amount of CO<sub>2</sub> absorbed by each plant when the chambers contained moist air was then measured for a period of three hours. Air in the chambers was then dried with CaCl<sub>2</sub> and a relatively low moisture content was maintained for a period of 15 hours, while the soil around the roots was supplied with a surplus of moisture. Measurements made during the last three hours of the period showed an average increase of approximately 25 per cent in the amount of CO<sub>2</sub> absorbed per hour by the plants. During the experiment the plants appeared to

be equally as turgid as similar plants which were kept in the greenhouse. The leaves were immediately severed from the petioles,

TABLE V  
WATER CONTENT OF PELARGONIUM LEAVES GROWN AT DIFFERENT  
TEMPERATURES AND RELATIVE HUMIDITIES

PLANT	PERCENTAGE RELATIVE HUMIDITY	TEMPERATURE (C.°)	VAPOR PRESSURE DEFICIT MM. HG.	PERCENTAGE LEAF MOISTURE
AFTER 3-HOUR EXPOSURE TO HIGH HUMIDITY AND TEMPERATURE OF APPROXIMATELY 26° C.				
13.....	55-60	25.0-26.0	10.3	89.5
14.....	60-63	25.5-26.0	9.5	88.7
15.....	58-60	26.0-26.0	10.2	89.8
16.....	65-65	25.0-25.0	8.2	90.2
17.....	65-70	25.8-26.0	8.0	88.4
18.....	63-63	26.2-26.5	9.4	89.5
Average.....	62	25.8	9.3	89.4
AFTER 15-HOUR EXPOSURE TO LOW HUMIDITY AND TEMPERATURE OF APPROXIMATELY 26° C.				
1.....	3-5	25.5-26.0	23.6	88.4
2.....	3-3	26.2-26.5	24.5	89.4
3.....	5-10	25.8-26.5	22.9	89.6
4.....	3-10	26.0-26.2	23.6	89.6
5.....	10-13	27.0-27.3	23.5	89.2
6.....	7-15	25.8-26.0	22.0	89.0
Average.....	7	26.2	23.4	89.2
AFTER 3-HOUR EXPOSURE TO LOW HUMIDITY AND TEMPERATURE OF APPROXIMATELY 40° C.				
7.....	14-14	39.0-40.0	46.3	88.3
8.....	15-16	40.0-40.8	47.4	88.4
9.....	18-20	40.0-41.0	45.6	87.7
10.....	20-20	40.5-41.0	45.7	87.6
11.....	17-18	40.0-40.8	46.3	88.3
12.....	30-31	40.0-39.8	37.9	88.0
Average.....	19	40.2	44.9	88.1

weighed in weighing bottles, and after drying in a well aerated oven at 75° C., their moisture content was determined (table V).

BRILLIANT (1) has reported that the leaves of *Hedera helix* and *Impatiens parviflora* absorb carbon dioxide most abundantly when their water content is below maximum. The slight increase in the average amount of carbon fixed by the plants of the present experiments during prolonged exposures to dry air was not the result of desiccation, however, as the water content of the leaves remained approximately constant.

Results of this experiment show that the rates of carbon fixation by turgid leaves of silver-leaved *Pelargonium* were not reduced either

TABLE VI  
AVERAGE AMOUNT OF CO<sub>2</sub> ABSORBED BY LEAVES OF  
PELARGONIUM UNDER VARIOUS CONDITIONS OF AT-  
MOSPHERIC MOISTURE AND TEMPERATURE

RELATIVE HUMIDITY (10-30%)		RELATIVE HUMIDITY (60-80%)	
TEMPERATURE (C°.)	UPTAKE MG. PER PLANT PER HOUR	TEMPERATURE (C°.)	UPTAKE MG. PER PLANT PER HOUR
26	4.6	25	5.8
30	6.1	28	6.1
34	4.9	33	5.6
38	4.3	36	4.3
41	3.7	38	4.1

by a rapid decrease in the humidity of the air surrounding the plants nor by prolonged exposures of 15 hours to low humidity. This result seems logical, for although low humidity inhibits the vegetative growth of many kinds of plants, the accumulation of carbohydrates which often accompanies this response indicates that the process of photosynthesis is not greatly retarded (5, 9, 15, 18, 20).

In other experiments with silver-leaved *Pelargonium* the rate of carbon fixation was measured while the plants were surrounded by air at various temperatures and containing different amounts of moisture. A group of six plants was exposed to a relative humidity of 10-30 per cent. The temperature was then increased slowly from about 25° to approximately 40° C., during which time changes in the rate of carbon dioxide absorption were recorded. Another group of

six plants was subjected to a relative humidity of 60-80 per cent and a similar increase in air temperature, while variations in the rates of carbon dioxide uptake were measured (table VI).

The plants exposed to a gradually increasing temperature and low humidity showed an increased rate of carbon fixation, which reached a maximum at about 30° C. and then decreased as the plants

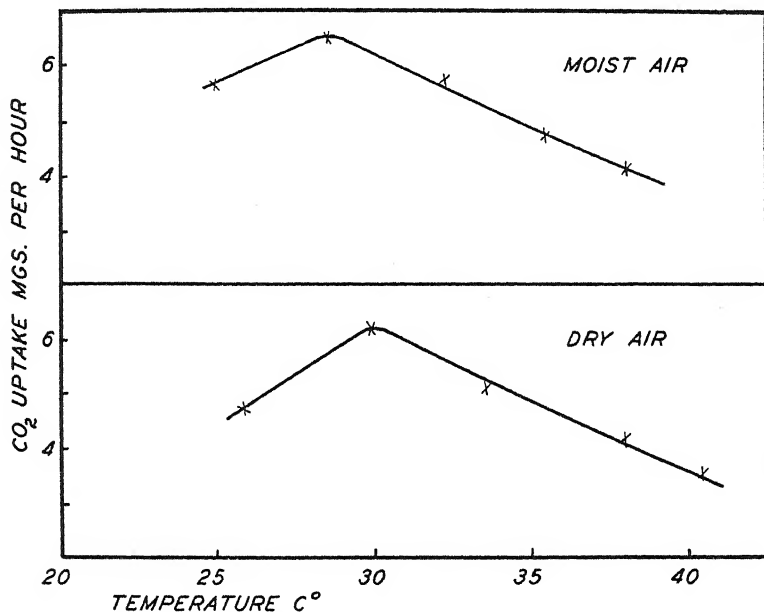


FIG. 2.—Graphs showing temperature at which maximum rate of carbon fixation occurred in group of *Pelargonium* plants subjected to high humidity (60-80 per cent) as compared with that of another group subjected to low humidity (10-30 per cent).

were subjected to higher temperatures. The rate of carbon fixation by similar plants which were subjected to the same temperatures, but surrounded with relatively moist air, also reached a maximum at approximately 30° C. and then decreased at higher temperatures (fig. 2). The moisture content of leaves subjected to relatively dry air and high temperatures was approximately the same as the moisture content of leaves under optimum conditions of temperature and humidity (table V). It is evident that such a decrease in the rate of carbon fixation was associated with high temperature rather than with desiccation of the leaves.

## EFFECT OF ATMOSPHERIC MOISTURE ON STOMATAL APERTURE

In the course of the foregoing experiments, it was often noted that the stomata of the plants exposed to low atmospheric humidity were generally closed, even though the roots were abundantly supplied with moisture. Also statements based largely upon observations of plants growing under natural conditions indicate that when atmospheric humidity is high, stomata open wider and remain open longer than when it is low (13, 15, 16). It was decided, therefore, to study the situation in more actual detail under controlled environmental conditions when the relative humidity of the atmosphere was varied and the temperature maintained at 25° C. In none of the experiments was there an apparent loss of turgidity of the leaves, although the extent of the stomatal opening studied generally decreased with a decrease in the moisture content of the surrounding air, and the stomata finally appeared closed when the leaves were subjected to relatively dry air. The size of the stomatal openings was determined at different periods and at different relative humidities during the course of the experiments by direct microscopic observation of the surfaces of the leaves, by measuring the width of the openings by means of a micrometer ocular without removal of the epidermis from the leaf, and by a study of collodion membranes or molds made according to the method described by LONG and CLEMENTS (14). Consistent results were obtained by all these methods.

As test plants the silver-leaved *Pelargonium* and tomato were used. The stomatal opening was measured as accurately as possible under the several conditions imposed. That carbon dioxide was fixed by the leaves was determined by three separate methods: (1) comparison of the amount of carbon dioxide absorbed by leaves under conditions in which the stomata were open and known to be favorable to photosynthesis with the amount absorbed by the same leaves when exposed to a dry atmosphere and with the stomata closed during the interval the photosynthetic measurements were made; (2) depleting the leaves of starch in the dark, as indicated by the iodine test, and then noting that an abundance of starch reappeared when the plants were illuminated in a relatively dry atmosphere even though the stomata were closed during the period of such exposure; (3) comparison, by means of chemical analyses, of the

carbohydrate content of leaves of plants illuminated in relatively dry air with the stomata apparently closed with the content of similar leaves in moist air having the stomata open.

#### RESULTS FROM COMPARATIVE METHODS

FIRST METHOD.—The amount of carbon dioxide absorbed by six plants when surrounded by moist air was first measured hourly for three hours. The relative humidity of the air around three of the plants was then reduced rapidly, while the moisture content of air

TABLE VII

CARBON DIOXIDE ABSORBED BY PELARGONIUM AND TOMATO PLANTS WHEN FIRST IN MOIST AND THEN IN RELATIVELY DRY AIR. RESULTS EXPRESSED AS MILLIGRAMS OF CARBON DIOXIDE ABSORBED PER HOUR PER PLANT

Hour	PERCENTAGE RELATIVE HUMIDITY			
	TOMATO		PELARGONIUM	
	70-90	20-30	55-75	10-15
1st.....	8.1	7.5	3.9	4.3
2nd.....	7.5	7.7	4.5	4.1
3rd.....	8.1	7.2	4.6	4.6
Average.....	7.9	7.5	4.3	4.3

surrounding the remaining plants was unchanged. The amount of carbon dioxide absorbed during the following three hours under these conditions was approximately equal to the amount absorbed during the previous three-hour period when the plants were exposed to a more humid atmosphere (table VII). Following these measurements, the plants were removed individually and the stomata quickly observed microscopically. The time required for this observation was less than three minutes, during which period no appreciable change occurred in the size of the stomatal openings. The stomata of leaves on plants exposed to low humidities were apparently closed while those on the leaves of plants exposed to high humidities were open (table VIII, fig. 3). This experiment was repeated, using several

groups of tomato and geranium plants, with consistent results. In general the maximum size of stomatal opening occurred when the leaves were exposed to relative humidities above 60 per cent. The size of the openings decreased with a decrease in humidity, and the stomata generally appeared closed in leaves exposed to relative humidities below 30 per cent. It should be stressed, however, that gases such as carbon dioxide, oxygen, and water vapor may pass between the guard cells of stomata although they appear microscopically to be pressed tightly together.

TABLE VIII

SIZE OF STOMATAL OPENINGS IN LEAVES OF PELARGONIUM AND TOMATO PLANTS GROWN AT DIFFERENT ATMOSPHERIC HUMIDITIES. RESULTS EXPRESSED AS AVERAGE WIDTH OF 25 STOMATA CHOSEN AT RANDOM, USING SEVERAL LEAVES

	PERCENTAGE RELATIVE HUMIDITY						
	PELARGONIUM			TOMATO			
	75	50	10	80	50	30	10
Width in mm. . . .	0.04	0.01	Closed	0.03	0.02	Closed	Closed

SECOND METHOD.— It was demonstrated by means of microchemical tests that leaves of the plants studied readily fixed carbon dioxide when in relatively dry air and with the stomata apparently closed. In these experiments the leaves of the plants were depleted of starch in the dark. They were then inclosed in chambers and exposed to arc light. Some of the plants were subjected to a relative humidity of 25 per cent, while others were given a relative humidity of 50 per cent. Microchemical examinations made during the following four hours showed the stomata of leaves at a high humidity to be open, while those of leaves at the low humidity appeared to be closed. At the end of four hours of illumination the chlorophyll was extracted with hot alcohol and the leaves tested for starch with the usual iodine test. Starch was stored in the illuminated leaves regardless of differences in humidity or size of the stomatal apertures (fig. 4).

THIRD METHOD.—The results presented are based on tests made by analyzing the leaf tissue of *Pelargonium* and determining the amount of acid-hydrolyzable materials immediately following a period of darkness during which time the starch was completely digested, as indicated by an iodine test. Plants used in these analyses are referred to as initial plants. The plants were then divided into

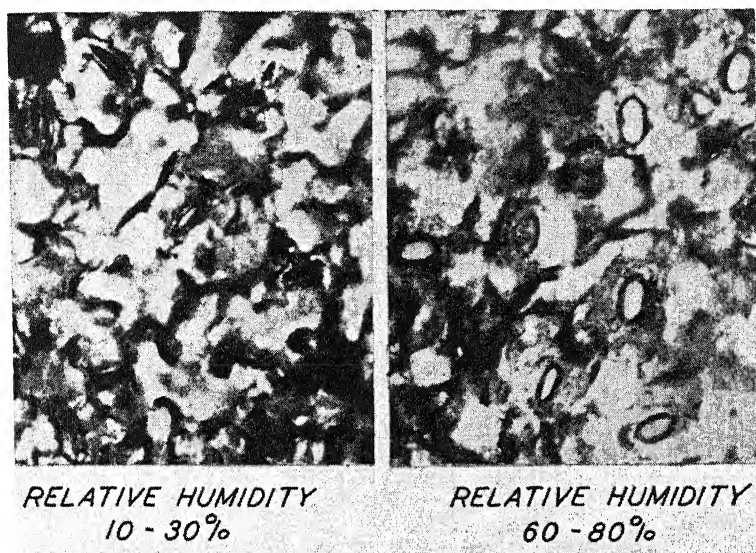


FIG. 3.—Photomicrograph showing size of stomatal apertures in *Pelargonium* leaf exposed to relatively dry air (left), as contrasted with size of stomatal apertures in another leaf of same plant when surrounded by relatively moist air.

two lots and placed under illumination, some of them being exposed to relatively moist and others to relatively dry air. At the end of a six-hour period of illumination, leaves from both lots were again analyzed and the results compared with the initial determinations.

During the interval of illumination the stomata of leaves subjected to a humidity of 10-30 per cent appeared to be closed, but the amount of acid-hydrolyzable materials in these leaves increased approximately 41 per cent over that of the initial plants. Stomata of leaves exposed to a humidity of 60-80 per cent were open and the amount of acid-hydrolyzable materials in these leaves increased



approximately 59 per cent during the period of illumination. It should be stated that results based on the method of carbohydrate analyses used do not, of course, represent all of the compounds of carbon formed in the leaves, and furthermore do not account for carbohydrates which were translocated to other portions of the plants during the period of illumination. It is evident from the results presented, however, that the amount of carbohydrate repre-

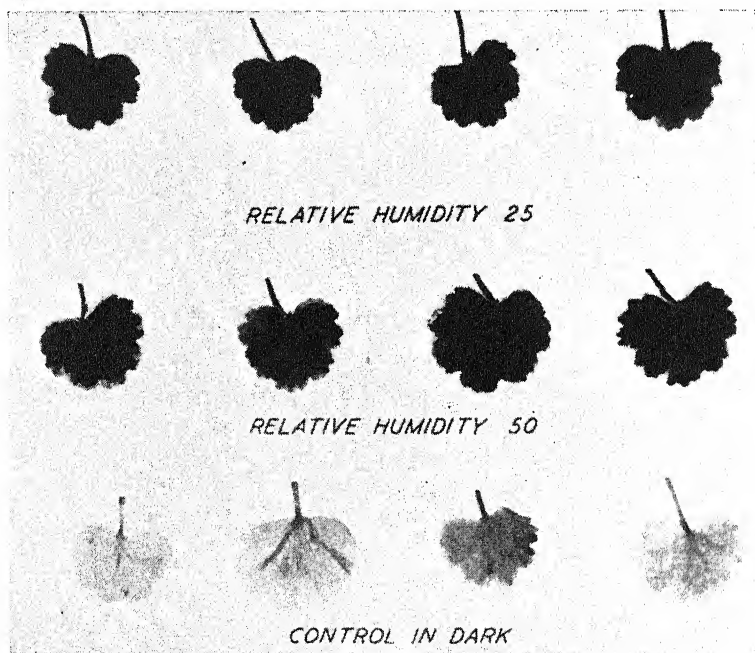


FIG. 4.—Showing starch synthesized by *Pelargonium* leaves at different humidities as compared with other leaves free of starch. The chlorophyll was extracted from the leaves and the starch stained with iodine.

sented in the acid-hydrolyzable fraction, such as sugars, starch, hemicellulose and other polysaccharides, increased in leaves grown in relatively dry air with the stomata apparently closed.

#### Summary

1. Rates of carbon fixation by leaves of squash, wax bean, cabbage, *Pelargonium*, *Primula*, and tomato plants were not affected either by a rapid decrease in the humidity of the surrounding atmosphere or by a prolonged exposure of 15–20 hours to low humidity.

2. Attached leaves of squash, wax bean, cabbage, *Pelargonium*, *Primula*, and tomato plants remained turgid at moderate temperatures although subjected to an extremely low humidity. The leaves of *Cineraria* plants in some few cases wilted when subjected to dry air, and the loss of turgidity was always accompanied by a decrease in the rate of carbon fixation.

3. Air temperatures above 30° C. retarded the rate of carbon fixation by *Pelargonium* leaves in either moist or dry air.

4. Atmospheric moisture affected the opening of the stomata of tomato and *Pelargonium* leaves. Under the conditions used, the maximum size of stomatal opening generally occurred when the leaves were exposed to relative humidities above 60 per cent. The size of the openings decreased with a decrease in atmospheric humidity and the stomata generally appeared closed in leaves exposed to humidities below 30 per cent.

5. The process of carbon fixation was not greatly affected by wide variations in the size of stomatal openings. The leaves of tomato and *Pelargonium* plants absorbed carbon dioxide and accumulated carbohydrates in appreciable quantities although the stomata appeared to be closed. That these plants absorb carbon dioxide when the stomata appear closed has been demonstrated by means of three methods: (1) the amount of carbon dioxide absorbed by leaves in which the stomata appeared closed was approximately equal to the amount absorbed by the same leaves when the stomata were open; (2) starch was found by means of the usual iodine test to accumulate in leaves exposed to relatively dry air with their stomata apparently closed; (3) acid-hydrolyzable carbohydrates in leaves of *Pelargonium* plants grown in relatively dry air increased approximately 41 per cent during a period of six hours, although the stomata of the leaves appeared to be closed.

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# FLORAL DEVELOPMENT AND VASCULAR ANATOMY OF THE FRUIT OF *RIBES AUREUM*

TSU KIANG YEN

(WITH THIRTY-SEVEN FIGURES)

## Introduction

*Ribes aureum*, the golden flowered currant, is closely related to *R. grossularia*, which GREW (7) has figured, showing the ovary as having two placentae, eight vascular bundles arranged as a ring in the fruit wall, and the enlarged cells of the seed coat. MALPIGHI (10) indicated that the structure of the gooseberry resembled the pomegranate and that each seed had a similar coating of juice cells. EICHLER (4) has given some of the taxonomical characters of *R. aureum*. VELENOVSKY (15, 16) mentioned the pericladium of *Ribes*. POHL (11) studied the fruit structures of many species of *Ribes*, and special attention was directed to the arillus of *R. aureum*. BUGNON (2) observed the trifascicular foliar trace of *R. sanguineum*, which resembles that of *R. aureum*. The present study of *R. aureum* deals with the development of the floral structures as well as with the anatomy of the fruit.

MATERIAL AND METHOD.—Material was collected on the campus of the University of Chicago from the autumn of 1929 until the autumn of 1932, and included series of buds and flowers, together with several stages in fruit development. The usual histological methods and stain technique were followed.

## Investigation

### DEVELOPMENT OF FLORAL ORGANS

The floral development of a number of plants belonging to the Rosoideae has been studied. GOFF (5, 6) studied the cherry, plum, apple, pear, and also called attention to the currant. He found that the flower primordia of the currant were just beginning to appear on July 8 at Madison, Wisconsin.

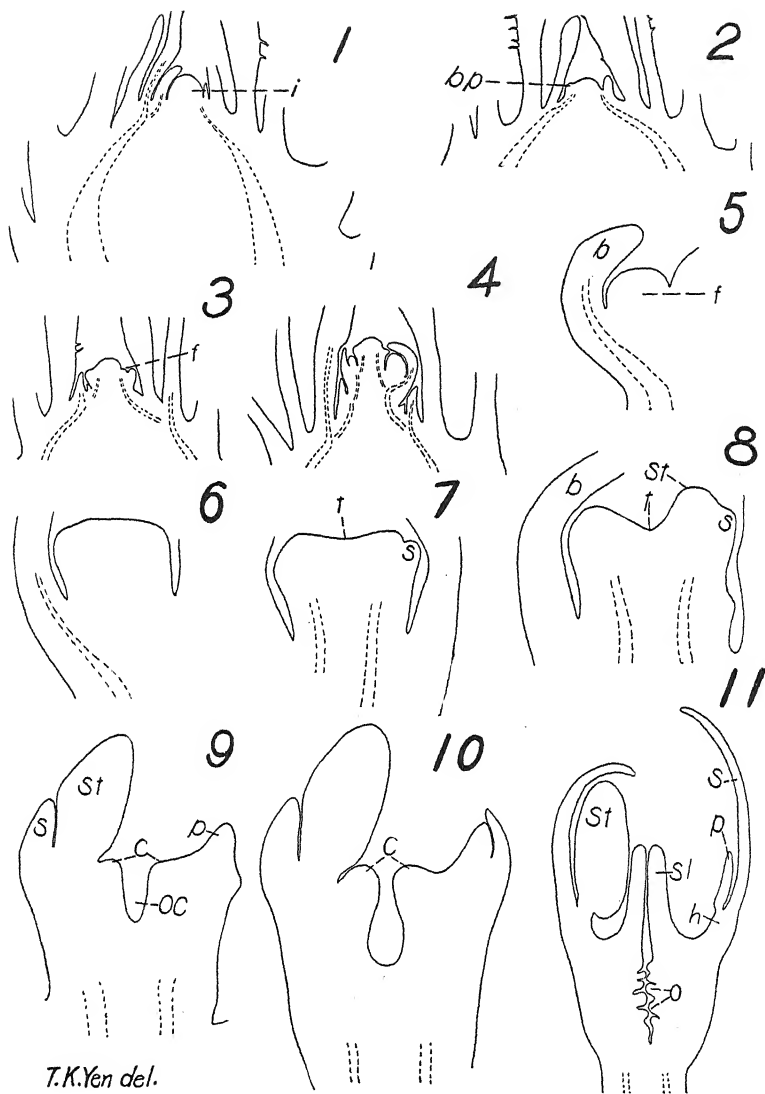
The differentiation of the floral primordia of *R. aureum* is some-

what similar to that of the apple, discussed in detail by BRADFORD (1), KRAUS (9), TUFTS and MORROW (14), and others. The outer bud scales of the dormant bud are five to seven cells in thickness; the epidermis is strongly cutinized, and on the adaxial surface many unicellular hairs are present. Nearly all the epidermal cells, except the hairs, contain tannin. A longitudinal section through a young bud collected August 2, 1931, shows a dome-shaped terminal meristematic portion surrounded by the primordia of spirally arranged bracts or scales. Below this tip the majority of the cells are parenchymatous, although it is possible to discern the structures usually present in a leaf bud (fig. 1).

From the terminal portion of the axis there is developed laterally the more or less ovoid primordium of a bract and soon thereafter a flower primordium arises in its axil. In like manner, bracts with floral primordia in their axils arise acropetally until each inflorescence is composed of six to eleven flower buds, the terminal one always remaining the smallest.

The first appearance of the individual flower is a dome-shaped protuberance (figs. 2-5). The growth of the young primordium is rather more rapid in circumference than in length. After reaching considerable size, elongation of the tip of the axis becomes progressively slower and finally ceases. Meanwhile the tissue near the periphery of the upper portion of the primordium grows more rapidly, and thus the primordium becomes much broadened (fig. 6).

SEPALs.—Five small protuberances are the first structures to arise from the outer circumference of the upper portion of the broadened axis. These are the sepal primordia (fig. 7). They grow into somewhat flattened, elongated structures (fig. 11). Differentiation and development of the epidermal cells are unlike on the two surfaces of the sepal. Those on the abaxial surface grow much larger, are covered by a thick cuticular layer, and some of them mature as well developed guard cells. Those of the adaxial surface have a very thin cuticular layer, are short and hair-like, and are responsible for the velvety appearance of the sepals (3, 13). The mesophyll tissue of the young sepal is composed of three or four layers of cells. They continue to divide until one or two more layers have been added. The cells of a layer which is next to the lower epidermis are rather



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FIGS. 1-11.—Fig. 1, longisection of bud collected August 8, 1930; *i*, primordium of inflorescence. Fig. 2, same of bud collected August 8, 1932 showing differentiation of bract; *bp*, primordium of bract. Fig. 3, same of bud collected August 8, 1932 showing differentiation of flower primordium (*f*). Fig. 4, same of inflorescence collected August 22, 1930 showing later stage of fig. 3. Fig. 5, longisection through single flower primordium; *b*, bract; *f*, flower primordium. Fig. 6, same of flower primordium showing broadening. Fig. 7, same of flower primordium showing beginning of cup-like cavity brought about by slower growth of tip of axis than surrounding tissue; *s*, sepal primordium; *t*, tip of axis. Fig. 8, same of later stage than fig. 7 showing differentiation of stamen primordium (*st*). Fig. 9, later stage than fig. 8 showing differentiation of petal primordium (*p*); *c*, carpels; *oc*, ovarian cavity. Fig. 10, later stage than fig. 9 showing centripetal growth of tissue around ovarian cavity. Fig. 11, longisection of young flower bud; *o*, primordia of ovules; *st*, stamen; *sl*, young style; *h*, hypanthium; *p*, petal; *s*, sepal. (Dotted lines show vascular bundles.)

compactly arranged, and plastids may be found in them. The cells of the other layers become parenchymatous, with large intercellular spaces.

**STAMENS.**—Immediately after the appearance of the primordia of the sepals, a ring of tissue arises just inside them. Soon five protuberances differentiate from the inside of the ring, each being opposite a sepal primordium. They grow upward and soon exceed the sepals in length. These young stamen primordia are ball-like at first (fig. 8) but grow rapidly and elongate (fig. 9). Some of the hypodermal cells divide periclinally and give rise to a single layer of primary sporogenous cells and one layer of primary parietal cells. The wall cells divide several times, resulting in three layers of wall cells and one layer of tapetal cells. The sporogenous cells divide several times and are in the pollen mother cell stage during the winter.

The next spring, microspores are formed. The outermost layer of the wall differentiates as the endothecium. The tapetal cells grow rapidly, soon exceed the wall cells in size, and then begin to degenerate. Before the dehiscence of the anther, they are absorbed completely. The pollen grains are round and smooth, with many germ pores on the surface. Disintegration of the cells between the two loculi begins after the maturation of the pollen grains, and the anther opens as soon as the disintegration of the septum is completed.

**PETALS.**—The order of development of floral parts is the same as found by SCHERTZ (12) in *Scrophularia*. As soon as the sepals and the stamens have been laid down, the primordia of the petals begin to differentiate (fig. 9). The rudiments of the petals appear similar to those of the stamens and are differentiated from the distal portion of the same ridge of tissue, alternating with the stamens. Three distinct cycles of primordia are now discernible, the stamen cycle on the inside, that of sepals on the outside, and between these two the petal cycle (figs. 9, 10). At this stage the rudiments of the petals are much smaller than those of either the stamens or the sepals. The rounded protuberances soon become ellipsoidal in cross section, and with the exception of the narrower epidermal cells, there are no noted differences between the cells composing them. The basal part of the petal primordia is made up of three or four layers of mesophyll cells, while at its apex only one layer is present. The epidermal cells

of both dorsal and ventral surfaces develop into short, hair-like papillae. At first the petals grow slowly in length (fig. 11), but a short time before the opening of the flower, elongation takes place rapidly and they reach their full length, roofing over the more centrally placed structures. The undiverged basal portions of the sepals, petals, and stamens form a tube, the hypanthium.

CARPELS.—At approximately the time the primordia of stamens and petals begin their development, those of the two carpels are initiated. These appear first as two oppositely placed blunt protuberances just within the cycle of stamens arising from the margin of the cup-shaped receptacle and some distance from the tip of the main floral axis, which is still slowly elongating and enlarging. Soon the whole inner margin of the receptacle grows (figs. 7, 8) and is elevated actively, as is also the tip of the axis except at the central portion and two small areas adjacent to it. The continued elevation of these portions, together with that part of the receptacle lying exterior to them, results in the formation of two communicating carpellary cavities (fig. 9), roofed over by the upper portion of the carpels and surrounded laterally by the ovary walls, the abaxial portions of which, however, are indistinguishable from the tissue of the receptacle from which they remain completely undiverged. The two protuberances first to arise elongate much more rapidly than do the remaining portions of the ovary, and extend upward as development takes place, completely diverged from the remaining portions of the receptacle, sometimes referred to as the calyx tube, and also from each other, except at the base.

WALL OF FRUIT.—A cross section through a very young flower primordium shows practically no differentiation among the cells of the wall of the fruit. All are meristematic. Later procambial strands are readily visible, the vascular bundles appearing closer to the periphery of the receptacle than to the ovarian cavity. The difference between the development of the epidermal cells on the abaxial and adaxial surfaces of the fruit is striking. Those on the outside divide actively. Some of them differentiate as guard cells; others soon become thick walled. A cuticle of considerable thickness is deposited on the surface of the fruit. The epidermal cells on the inside of the ovary wall do not divide so actively, but elongate mainly tangential-



ly. A thin layer of cuticle is observable on them, but later the walls disintegrate and a sheath of gelatinous matter is present in the ripe fruit.

Some of the cells scattered among the parenchymatous tissue of the fruit wall grow more rapidly than others, and chloroplasts develop in them. The walls of these cells become thicker, but after pollination the chloroplasts gradually degenerate, and in a section of the ripe fruit no differences between these cells and the rest can be recognized.

PLACENTA AND OVULES.—As noted previously, there are two carpels, undiverged at their edges up to the time of pollination. These edges develop relatively little toward the center of the axis and thus appear as parietal placentae on opposite sides of the ovarian cavity. During development and maturation of the fruit, they increase in size appreciably. Several rows of ovules arise from them (fig. 11). The inner integument differentiates first but soon is overlapped by the more rapidly growing outer integument. An arillus develops from the funiculus of each ovule. No indication of the arillus can be observed until the diameter of the young fruit exceeds 2 mm. As the fruit grows from 2 to 3 mm. in diameter, the funiculus begins to increase both in number and in size of cells, finally almost enveloping the entire seed. In a section of a ripe fruit, the arillus appears as the pulp of disintegrated parenchymatous cells which fills up most of the space around the seeds. POHL (11) has described the arillus of this fruit.

STIGMA AND STYLE.—How the primordia of the carpels arise has been stated previously. A zone of cells surrounding the stylar canal remain meristematic and serve in part as conductive tissue. The epidermal cells of the most distal, stigmatic surface elongate into papillae. Some of the cells underlying the surface disintegrate a short time before blooming of the flower and form a mucilaginous mass at the surface.

After pollination, the upper portion of the style withers. Following this, two proliferating masses of tissue arise opposite each other on the inner portion of the base of the style. Each is derived from two adjacent carpels. The cells are at first parenchymatous but become suberized during the ripening of the fruit. The proliferations are not

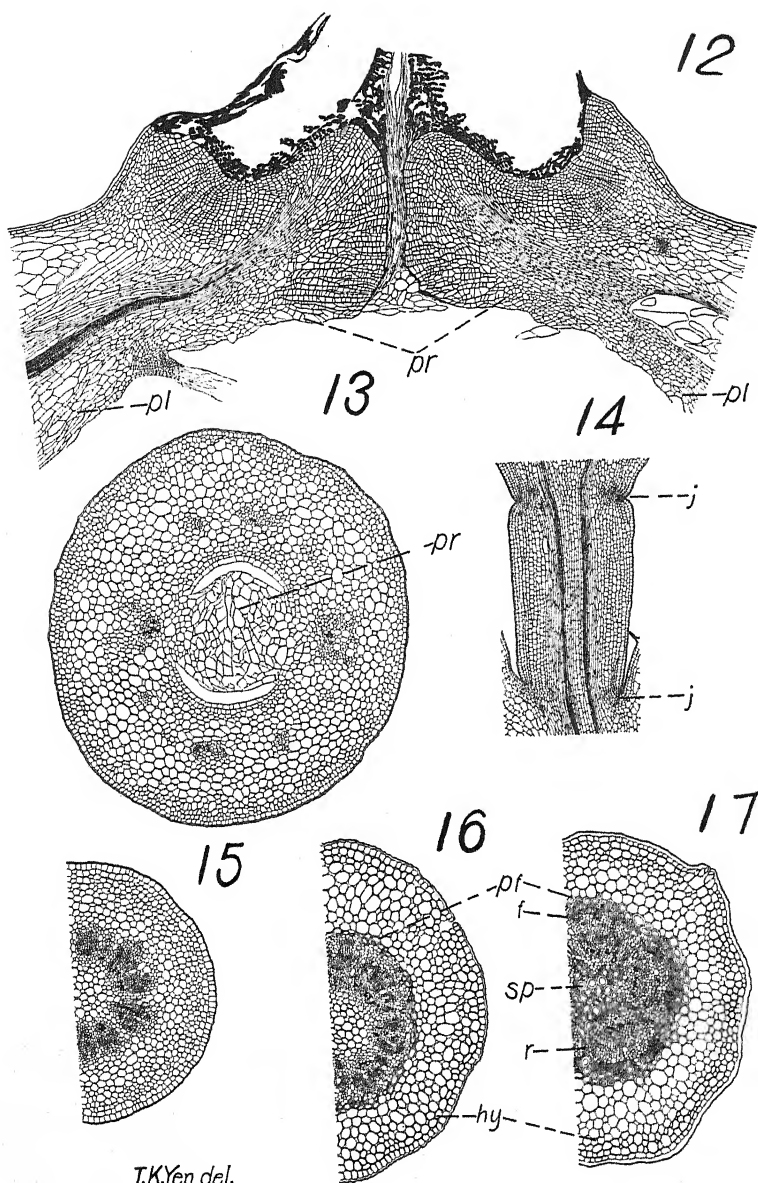
connected with the placentae, although they appear somewhat similar (fig. 13). These two masses of tissue develop rather late. In a very young fruit with a diameter of 3 mm., no trace of the proliferation is observable. When the fruit reaches a diameter of 4 mm., the cells on the inner margin of the base of the style begin to show divisions. The formation of these proliferations is completed in a few days. A cross section through the basal portion of a fruit 5 mm. in diameter shows the stylar cavity almost filled by them (fig. 12).

NECTARY.—The nectary is located in the groove between the hypanthium and the base of the style. In a transverse section of a young flower, it appears first as an active layer of cells next to the epidermis. These cells first divide tangentially but later both tangentially and radially. This activity results in the formation of a ring of tissue consisting of small cells with dense cytoplasm. In a ripe fruit the walls of these cells are suberized.

#### VASCULAR ANATOMY

BRACT AND PEDICEL.—The vascular bundles in the axis below the base of the inflorescence are arranged in a ring (fig. 18); individual bundles at this point are not clearly distinguishable and are indefinite in number. At a higher level the ring is flattened out into an ellipsoid (fig. 19). Three bundles, one of which is situated at an extremity and the two others located near the middle of the ellipsoid, separate from the ring (fig. 20) and extend into the cortex. They are the main vascular bundles of the bract. This derivation of the three bundles of a bract is the same as in *Ribes sanguineum* as described by BUGNON (2). The lateral veins in the bract of *R. aureum* are more extensive and more profusely branched than is the midrib.

When these three bundles have diverged into the cortex, three gaps are left in the stelar ring (fig. 21), thus separating the ring into two small arcs on either side of the gap and a half circle opposite these two small arcs (figs. 20, 21). The two small arcs of vascular tissue soon diverge into the pedicel of the flower located in the axil of the bract. Above the point of divergence of the bract and the flower, the half ring of the main axis soon becomes a complete one (fig. 26), owing to the differentiation of additional xylem and phloem elements. In like manner this ring of vascular tissue is broken into the



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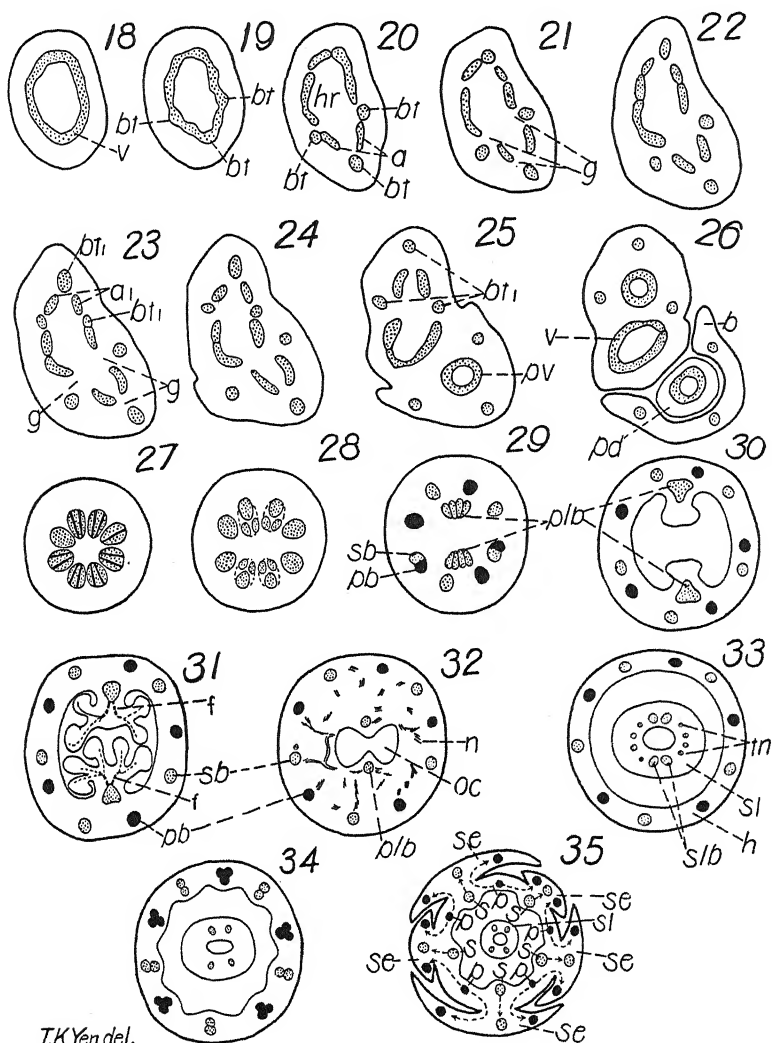
FIGS. 12-17.—Fig. 12, longitudinal view of upper portion of ripe fruit showing proliferations at basal portion of stylar canal; *pl*, placenta; *pr*, proliferations. Fig. 13, cross section of stylar region of young fruit with diameter of 5 mm., showing proliferations (*pr*). Fig. 14, longisection of young pedicel showing differentiation of two joint-like structures (*j*). Fig. 15, cross section of young pedicel showing parenchymatous tissues at that stage. Fig. 16, later stage than fig. 15 showing pericycle and phloem cells beginning to differentiate into fibers and the sclerotic condition of cells of hypodermis (*hy*); *pf*, pericyclic fibers; *f*, phloem fibers. Fig. 17, cross section through pedicel of ripe fruit; *sp*, sclerotic pith; *r*, sclerotic ray cells; *pf*, pericyclic fibers; *f*, phloem fibers.

half ring and two small arcs, which diverge into the flower pedicel, at each successive divergence of a bract and a flower (fig. 26). The differentiation of vascular elements in the pedicel of the flower above the point of divergence of the two small arcs soon closes the two gaps, separating the two arcs, and the ring of vascular tissue is complete.

The stelar ring of the pedicel is composed of eight collateral bundles arranged similarly to those in the stem (figs. 27, 37). Each bundle has from one to three rows of vessels separated by ray cells (fig. 27). Patches of smaller phloem cells separated by larger ray cells are present on the outside of each row of vessels.

**BUNDLES OF BASAL PORTION OF FRUIT.**—In that portion of the pedicel which flares out at the base of the fruit, the vascular bundles appear somewhat oblique. They are gradually extended toward the periphery of the fruit (figs. 28, 37), parenchymatous cells lying on all sides of them toward the central portion of the fruit as well as toward the outer epidermis. It is not possible to allocate this parenchymatous tissue either to a cortical or pith region since the cells appear so similar in general structure. The bundles are grouped into two crescent-shaped masses, each group composed of four bundles (figs. 28, 37).

**PLACENTAL BUNDLES.**—Of the four bundles composing the group just mentioned, the two central ones in turn are made up of three smaller bundles (figs. 27, 28). At a slightly higher level the central bundle of each of these groups of three is diverged slightly outward toward the periphery of the fruit, and of these two one is continued upward and finally terminates in a petal, the other in a sepal. The two remaining small bundles of each of these groups of three, making four in all (fig. 28), are diverged toward the carpellary cavity (fig. 29). They approach each other very closely and continue upward as the vascular system in one of the placentae (figs. 29-35). The other four bundles of the two groups lying at the tip of the crescents are not divided into smaller bundles at this level of the fruit (fig. 28), but extend toward the periphery of the fruit wall, so that at this level there are eight bundles in a circle at the periphery, and four placental bundles in two groups near the center (fig. 28). Each placental bundle is diverged into a number of lateral veins from which many vein-



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FIGS. 18-35.—Fig. 18, diagram of cross section of base of axis of inflorescence showing vascular cylinder (*v*) as complete ring. Fig. 19, diagram of cross section of axis of inflorescence slightly above that of fig. 18 showing differentiation of bract traces (*bt*). Fig. 20, same, a little above fig. 19, showing vascular cylinder broken into the three traces of bract (*bt*), three gaps, two small arcs (*a*), and a half ring (*hr*). Fig. 21, same slightly above fig. 20, showing the three gaps (*g*). Fig. 22, bract traces extending toward periphery. Fig. 23, differentiation of another bract and floral primordium slightly above fig. 22; *bt*, bract traces of another bract; *al*, small arcs of another primordium. Fig. 24, diagram a little above fig. 23 showing traces extending more to periphery of cortex. Fig. 25, two small arcs of first flower primordium becoming a ring; *pv*, vascular ring of pedicel. Fig. 26, diagram of cross section through node of inflorescence axis showing bract (*b*), pedicel (*pd*), and complete vascular ring of axis (*v*). Fig. 27, cross sectional

lets are derived. These veinlets are the vascular bundles to the ovules (fig. 31). The lateral veins of a main placental bundle are arranged in two vertical rows parallel to each other. The veinlets of the two rows remain distinctly separate and each placenta is therefore recognizable by its venation, as it were, as consisting of two halves (fig. 31).

**BUNDLES OF STAMENS, SEPALS, AND PETALS.**—At a level slightly higher than that at which the placental bundles become definitely recognizable, two of the peripheral bundles located at the extremities of one of the crescent-shaped groups are divided to form two bundles each (fig. 29). Of the four bundles resulting, two finally terminate in the petals, two in the sepals. Thus ten vascular bundles are formed in a ring near the periphery of the wall of the receptacle (fig. 30). These are the primary bundles of the sepals, stamens, and petals. Each bundle may be branched to form one or more small bundles near the top of the fruit. These small branched bundles extend centripetally almost perpendicularly to the main bundles, diverging freely to form a fine network crowning the upper portion of the ovary (figs. 32, 37). The centripetal differentiation of these veinlets progresses almost to the ovarian cavity and a ring of bundles is completed, with the addition of the placental bundles, around this cavity (fig. 37).

In the upper portion of the hypanthium before the divergence of the sepals, petals, and stamens, the bundles are arranged in two con-

view of pedicel showing eight vascular bundles arranged in ring, six with three rows of vessels, one with two rows, and one with one row. Fig. 28, diagram of pedicel showing eight bundles arranged in two crescent-shaped groups with two bundles in middle of each group dividing into three bundles each. Fig. 29, diagram showing union of four small bundles into placental bundle (*plb*) and two bundles at either extremity of one of the crescents dividing to form two bundles each. Dotted bundles in outer ring of diagram are stamen bundles (*sb*) and black ones are petal bundles (*pb*). Fig. 30, cross sectional diagram through lower portion of fruit showing two placental bundles (*plb*) and ten primary vascular bundles. Fig. 31, diagram of cross section through middle of fruit showing two parallel rows of vascular bundles (*f*) derived from placental bundles; *sb*, stamen bundles; *pb*, petal bundle. Fig. 32, diagram of cross section through upper portion of fruit showing network of bundles (*n*), ovarian cavity (*oc*), and placental bundle (*plb*). Fig. 33, diagram of cross section through hypanthium (*h*); *sl*, style; *tn*, tips of bundles of network; *sib*, stylar bundles. Fig. 34, cross section through upper portion of hypanthium showing divergence of bundles. Fig. 35, diagram through top of flower showing petals and stamens alternately arranged; *se*, sepals on outside, showing bundle derivation; *s*, stamens; *p*, petals; *sl*, style. Arrows show divergence of sepal bundles.

centric rings (fig. 34), those of the petals and stamens in an inner ring alternating with the sepal bundles. Five of the ten main bundles present here are common bundles of the stamens and sepals (fig. 34). These five usually do not branch until the middle part of the hypanthium is reached, where each branches into two (fig. 36),

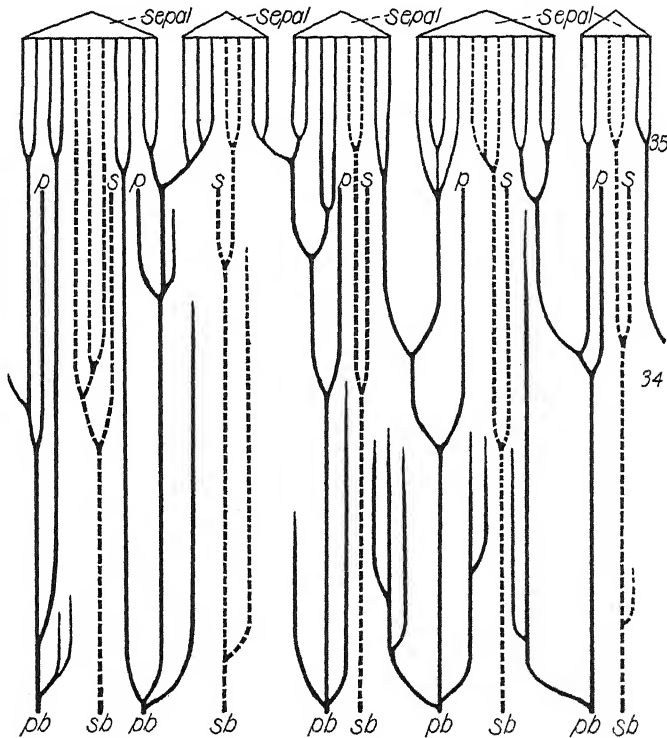


FIG. 36.—Diagram showing venation of petals (*p*), sepals, and stamens (*s*); *pb*, petal bundles; *sb*, stamen bundles.

one extending to the stamen, the other dividing again into two or occasionally more and extending to a sepal (figs. 35, 36). The single bundle which diverges into the stamen remains unbranched.

The other five main bundles terminate in the petals (figs. 34-36). They are arranged alternately between the bundles which are diverged into the stamens, are occasionally rebranched just above the level of the network near the top of the ovary, and these branches

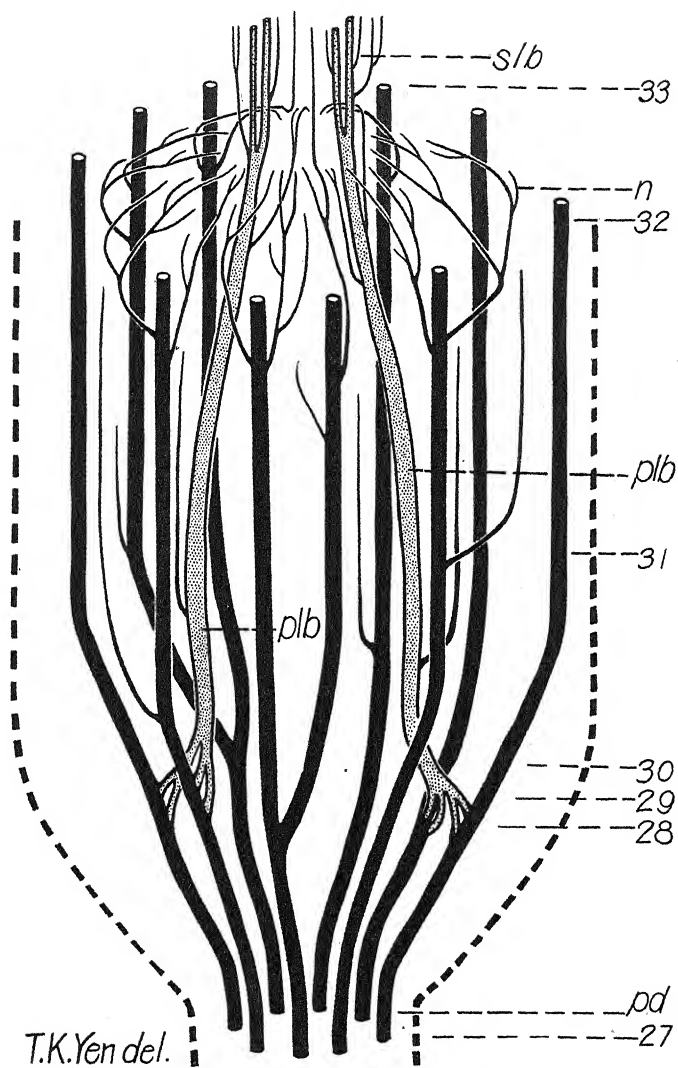


FIG. 37.—Diagram showing venation of a fruit: *s/b*, style bundles; *n*, network; *plb*, placental bundles; *pd*, pedicel. Small numbers at right of figs. 36 and 37 indicate places from which the cross section diagrams bearing similar numbers were made.



are diverged into the sepals. Each of these five main petal bundles is divided into two at a point half way up the calyx tube (fig. 36), one becoming the main vascular bundle of the petal. This bundle is rebranched into six or more veins; the other gives rise to two or more branches, each of which is divided into two veinlets which diverge into the lateral portions of either adjacent sepal (figs. 35, 36).

**BUNDLES IN STYLE.**—Near the base of the style the two placental bundles already described are diverged into two main bundles and several smaller ones. These small bundles are rebranched as a network in the tissue of ovarian walls. They extend through the entire length of the style and are divided into several small veinlets which are traceable almost to the tip of the stigma (fig. 35). Some veinlets other than those derived from the placental bundles are present on either side of the style. These are extensions of the branches from the network near the top of the fruit and are traceable for a short distance in the style above the ovary (fig. 33).

Comparing the floral and vascular anatomy of *Ribes aureum* with the apple (9) in the Rosaceae, the former seemingly represents an advance in the decreased number of stamens and carpels, in the lack of clear demarcation of the ovarian tissue from that of the receptacle, and in the failure of divergence of a distinct vascular supply for the carpels except as represented by the vascular system in the placentae and a portion of the vascular bundles of the styles.

### Summary

1. Except for the terminal flower, each flower primordium of *Ribes aureum* arises in the axil of a bract. It is a dome-shaped protuberance at first but soon becomes cup-shaped owing to the lateral expansion of the axis.
2. The order of the floral development is sepals, stamens, petals, and carpels.
3. The carpels are undiverged at the bases and two virtually parietal placentae are present.
4. The inner integument of the ovule is initiated first but is soon overlapped by the outer one. After fertilization, the epidermal cells of the outer integument enlarge and become succulent.
5. The funiculus enlarges as an arillus, the cells of which disinte-

grate into a pulpy mass almost filling the space surrounding the seeds.

6. The two placenta-like proliferations derived from either side of the base of the stylar canal have no connection with the placentae.

7. Two joint-like structures are found at either end of the pedicel.

8. The vascular bundles of the pedicel are eight in number. They divide to form two crescent-shaped groups in the fruit wall, each of four bundles.

9. A placental bundle is derived from the two bundles at the center of each crescent. Each placental bundle gives rise to two vertical rows of veins which are parallel and remain distinctly separate.

10. Each stamen bundle is diverged into two, one of which extends to the sepal and divides again, the other to the stamen and is undivided.

11. Each petal bundle is alternate with a stamen bundle. It is divided into two, one extending into the petal where it is divided again into three bundles with many veinlets; the other is divided into two, one of each of these diverging into the outer portion of either adjacent sepal.

12. The bundles of each sepal are derived from three different origins, those in the center from a bundle common with the stamen bundle for some distance, and those on either side diverged from adjacent bundles terminating in the petals.

13. The bundles of the style are derived primarily from the placental bundles. A few small bundles are diverged upward from the network of bundles at the top of the ovary.

14. The vascular bundles in the fruit wall are arranged in a single ring, which makes impossible a classification of them into carpellary and toral bundles or a segregation of the parenchymatous cells surrounding them into pith and cortex.

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# VASCULAR ANATOMY OF THE SEEDLING OF MELILOTUS ALBA

ELMER B. MCMURRY AND EMMA L. FISK

(WITH EIGHTEEN FIGURES)

## Introduction

The vascular anatomy of the seedlings in the species of the Leguminosae which have been studied has been found to be exceedingly variable. As white sweet clover has assumed increasing importance economically, it appeared that a study of the transition region in its seedling might prove of interest. The development of the embryo sac and the formation of the embryo in this species was traced by YOUNG (9). COOPER (2) gave an account of macrosporogenesis and early embryology in *Melilotus alba* and *M. officinalis*. Fertilization and the early development of the embryo were traced by COOPER in the variety Redfield Yellow.

Work on the root in the Leguminosae dates back to the classical studies of DE CANDOLLE, VAN TIEGHEM, NÄGELI, and others. According to VAN TIEGHEM (5), NÄGELI in 1858 referred to the centripetal development of the three or four xylem strands in the roots of *Baptisia* and *Lathyrus*. He pointed out that the arrangement of the vascular tissues in the root was the reverse of that of the stem. VAN TIEGHEM (5) stated that the anatomical limit of root and stem is the place where the change from the alternate to the superposed position of the phloem in relation to the xylem occurs, and where the xylem rotates from a centripetal to a centrifugal type.

VAN TIEGHEM (6) described the roots in a number of species in the Leguminosae, including *Medicago sativa*. In regard to the number of xylem strands in the roots in the family, he stated, "Quant au type numérique, il varie dans la famille; fixe quand il est de deux, trois, ou quatre." GERARD (3) referred to the structure of the seedling in *Medicago falcata*, a member of the Trifolieae. VAN TIEGHEM (7) observed that the protoxylem strands of the root migrate to form the many bundles of the stem of *Medicago*.

COMPTON (1) described *Melilotus arvensis* as having a triarch protostele in the root, which changes to diarchy in the hypocotyl by the disappearance of one of the protoxylem ridges. One of the three phloem groups divides to form a fourth phloem strand. He stated that the vascular supply to each cotyledon consists of a "tangential row of bundles, in the middle the vestigial protoxylem, on either side of this a wedge-shaped metaxylem group, and on the extremities of the row the phloems." COMPTON described briefly and figured stages in the structure of the seedling in several other members of the Tri-folieae, including *Ononis biflora*, *O. rotundifolia*, *Trifolium arvense*, *T. hybridum*, and several species of *Trigonella* and *Medicago*.

WINTER (8) has described the vascular anatomy of young plants of *Medicago sativa*. She stated, "each cotyledonary trace is made up of a polar xylem strand and a part of each lateral xylem strand" and "cotyledonary traces, as they pass into the cotyledons, are composed of triad bundles, the median portion being a polar protoxylem strand." The change to the collateral endarch bundle occurs in the midrib strand in each cotyledon.

MATERIALS AND METHODS.—Entire plants of *Melilotus alba*, varying from one to sixty days in age, were killed and fixed in Flemming's medium solution, Licent's fixative, Rawlins' fixative, or in chromo-acetic acid. Very satisfactory results were obtained with the use of butyl alcohol as a dehydrating and clearing agent. In the case of younger seedlings, serial sections of entire plants were used.

### Observations

#### SEED AND SEEDLING

The mature seed is characterized by a curved embryo in which the cotyledons and hypocotyl lie parallel with one another (2). The cotyledons are flat and leaflike at maturity and are filled with reserve foods. No endosperm is present in the mature seed.

The seedling is epigeal in development, in that an arched hypocotyl pulls the cotyledons above ground and the latter become photosynthetic. The blades of the cotyledons are oval in shape and the petioles reach a length of 1-2 mm. A unifoliate leaf with stipules develops next above the cotyledons; the third node is characterized

by a typical trifoliate leaf with stipules. A small branch arises early in the axil of the unifoliate leaf, followed by a second in the axil of the first trifoliate leaf. Normally the buds in the axils of the cotyledons do not develop into branches the first year.

#### PRIMARY ROOT

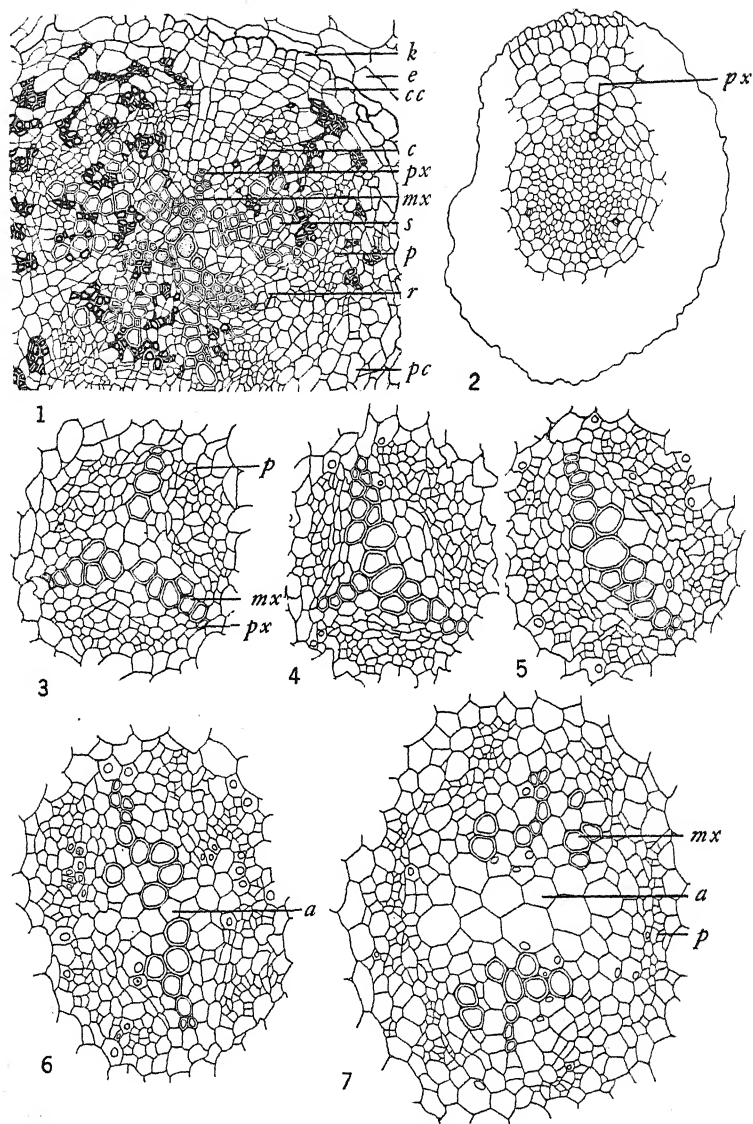
The primary root is characterized by a triarch protostele with three phloem strands alternating with the exarch xylem rays (fig. 3). The outermost cells of the phloem nearest to the protoxylem differentiate as fibers by the time the seedling is seven to nine days old. A pericycle one to two cells in thickness limits the stele; this in turn is surrounded by a row of endodermal cells characterized by Casparian strips. The cortex, which is only several cells in thickness, appears relatively inconspicuous. Secondary roots arise early in the pericycle opposite the protoxylem.

Cambial activity begins in the primary root when the seedling is seven to eight days old, and fibers differentiate early among the conducting elements of the xylem and phloem (fig. 1). This conspicuous development of fibers in the young root is found to be characteristic of a number of species in the Leguminosae. As cambial activity continues, conspicuous parenchymatous rays are differentiated opposite the three primary xylem ridges (fig. 1). A cork cambium arises early in the pericycle and the cortex becomes crushed and soon disappears. The older root is protected by a prominent layer of cork.

#### HYPOCOTYL

Serial sections from plants four to seven days old, in which the primary tissues were differentiating and in which there was little or no evidence of cambial activity, were used first in the study of the transition region. This was supplemented by a study of successively older plants up to three or four weeks in age, in which a considerable amount of secondary tissue was evident.

One of the first indications of a change from the typical triarch protostele of the root to the diarch condition in the hypocotyl is noted in the primary xylem elements. In many seedlings approximately four days old, at the base of the hypocotyl one of the xylem strands appears shorter than the other two (fig. 4); at successively



FIGS. 1-7.\*—Fig. 1, transverse section of portion of primary root from plant three weeks old, showing primary and secondary tissues;  $\times 170$ . Figs. 2-7, transverse sections from seedling four days old; fig. 2, primary root showing differentiation of protoxylem,  $\times 170$ ; fig. 3, triarch protostele of root showing differentiation of metaxylem and phloem,  $\times 360$ ; fig. 4, section at base of hypocotyl showing transition from triarchy to diarchy,  $\times 360$ ; fig. 5, diarch protostele at slightly higher level than in fig. 4,  $\times 360$ ; fig. 6, stele in upper hypocotyl showing parenchymatous cells among thick walled elements of xylem,  $\times 360$ ; fig. 7, lateral differentiation of metaxylem, increased number of strands of phloem, and development of pith in upper hypocotyl,  $\times 360$ .

\* All drawings were made with the aid of a projectoscope. The following abbreviations are used on all illustrations: *px*, protoxylem; *mx*, metaxylem; *s*, secondary xylem; *p*, phloem; *pc*, pericycle; *e*, endodermis; *c*, cambium; *cc*, cork cambium; *k*, cork; *r*, ray; *a*, parenchyma; *l*, collenchyma; *ax*, cotyledonary axis; *cot*, cotyledon; *ec*, epicotyl; *unt*, unifoliate leaf; *b*, bud; *m*, median trace; *la*, lateral trace.

higher levels fewer elements are differentiated in this strand and it ultimately disappears (fig. 5). This change is accompanied by a gradual orientation (fig. 4) of one of the other two protoxylem points into a position such that a typical diarch protostele results (fig. 5). At the levels where the diarch condition is evident, the stele appears oval in cross section. This diarch arrangement extends through the major portion of the hypocotyl.

In the upper hypocotyl, however, some of the elements of the metaxylem are oriented laterally with parenchymatous cells among them; a few metaxylem elements remain constantly in association with the protoxylem (fig. 6). At successively higher levels there is a gradual change in the position of the "migrating" elements of the metaxylem, until just below the cotyledonary node they appear in a position lateral to and on either side of the protoxylem points (fig. 7). These two primary groups of xylem elements form the traces to the cotyledons. The parenchymatous cells form a conspicuous pith, the stele appears considerably enlarged in transverse section, and the cells of the pericycle have increased in number (fig. 7).

Changes in the primary strands of phloem accompany the changes in the xylem. At the level in the lower hypocotyl at which one of the xylem strands becomes abortive, each group of phloem elements is flattened tangentially (fig. 4) and at higher levels is separated into two or more strands. In young seedlings at the diarch level there are usually evident on either side of the xylem plate three or four groups of small cells, which are apparently undifferentiated elements of the phloem (fig. 5). At higher levels those phloem strands nearest the protoxylem groups in the axial plane become associated with the adjacent xylem in the formation of the cotyledonary traces (fig. 7). Other groups of meristematic tissue in the stele mature later, as parts of the collateral bundles which are continuous with the bundles of the epicotyl.

A second type of reorientation of the xylem different from the preceding was found to occur in other seedlings with about equal frequency. In these, as serial sections at successively higher levels are examined, the earliest indication of transition in the hypocotyl is the differentiation of two of the protoxylem groups in a position to form the cotyledonary axis, and simultaneously several metaxylem ele-



ments differentiate opposite the third protoxylem group. At higher levels these metaxylem elements become more numerous and a tetrarch protostele results (fig. 8), with the rays on the cotyledonary and intercotyledonary axes at right angles to one another. However, spiral and annular elements characteristic of protoxylem are not recognizable in the new, fourth arm. At the levels where a tetrarch condition prevails in some plants, a few parenchymatous cells are found among the thick walled elements.

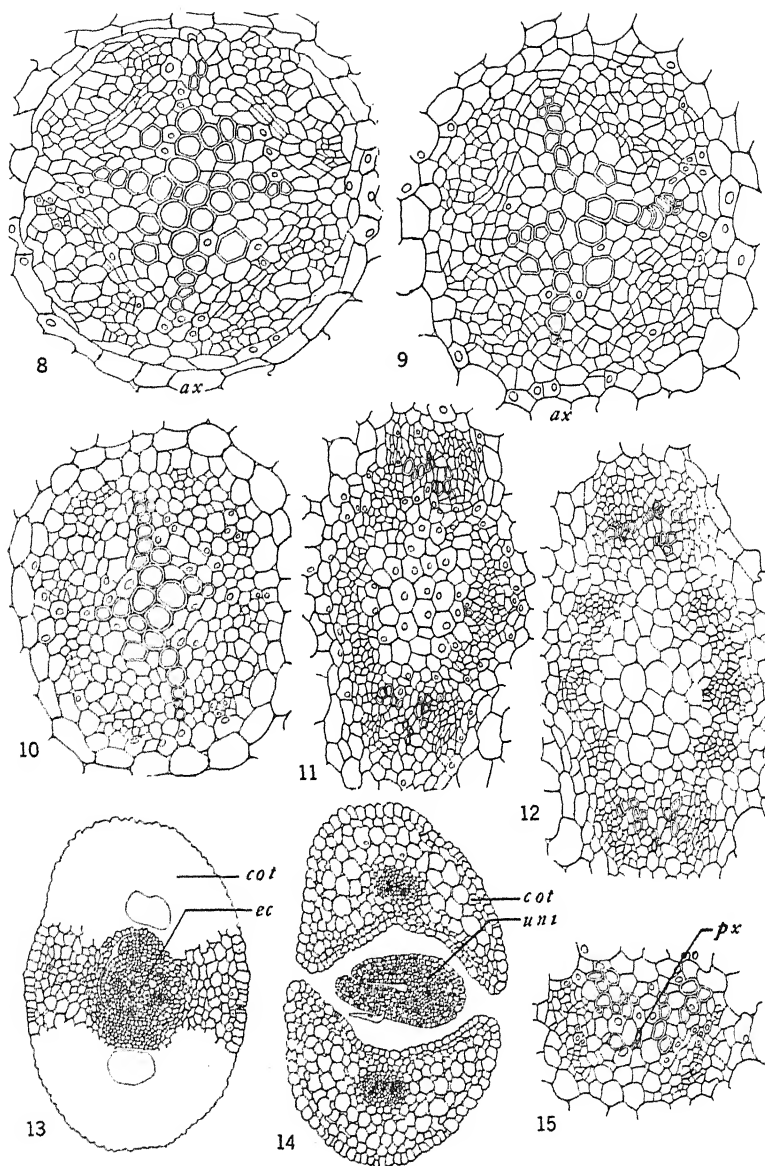
In this second type of seedling tetrarchy gives place rapidly at higher levels to diarchy, as fewer xylem elements differentiate in the last formed ray, accompanied by the lack of differentiation of the original exarch strand opposite (fig. 10). A diarch protostele results, or in some instances a diarch plate with numerous thin walled cells differentiating among the thick walled xylem elements. With the increase in number of cells in the pith at higher levels, the two polar groups with the less closely associated metaxylem elements now separate and become part of the traces to the cotyledons, as in the first type of seedling. The changes in the phloem of the tetrarch type in general appear to be similar to those in the seedlings first described.

Two plants were studied which show an intermediate condition between the two types described, in that only a weak fourth xylem ray differentiates (fig. 9). At a slightly higher level a distinctly diarch condition is noted.

#### COTYLEDONS AND COTYLEDONARY TRACES

The protoxylem strands in the axial plane, together with the associated metaxylem and groups of phloem, constitute the traces to the cotyledons. Each trace appears as two collateral strands made up of phloem and metaxylem, with several protoxylem elements between the two groups of metaxylem, thus approaching a mesarch condition (figs. 11, 12). Such a trace was described by THOMAS (4) as a "double bundle" and by COMPTON (1) as a "triad."

As each trace diverges from the stele, a gap in the meristematic cylinder is left (fig. 12). The cotyledons become distinct from the axis as an ensheathing tube. In seedlings several days old, the em-



FIGS. 8-15.—Fig. 8, transverse section of portion of lower hypocotyl from seedling seven days old, showing tetrarchy,  $\times 300$ ; fig. 9, transverse section from hypocotyl of another seedling showing weak development of fourth xylem arm,  $\times 300$ ; fig. 10, section of hypocotyl at level above that of fig. 8, showing reduction of xylem arms in intercotyledonary plane in transition from tetrarchy to diarchy,  $\times 300$ ; fig. 11, siphonostele in hypocotyl just below cotyledonary node in seedling four days old, differentiation of xylem of trace to epicotyl,  $\times 150$ ; fig. 12, divergence of cotyledonary traces from stele, early differentiation of six traces to epicotyl,  $\times 150$ ; fig. 13, differentiation of cotyledons from axis in seedling four days old and origin of cotyledonary buds,  $\times 90$ ; fig. 14, section of seedling four days old showing petioles of cotyledons and blade of unifoliate leaf,  $\times 90$ ; fig. 15, enlarged bundle of cotyledonary petiole, showing collateral arrangement of phloem and metaxylem strands, with protoxylem between the metaxylem groups approaching an endarch condition.

bryonic cotyledonary buds may be distinguished at this level (fig. 13). The cotyledonary petioles separate from each other on one side at a lower level than on the other.

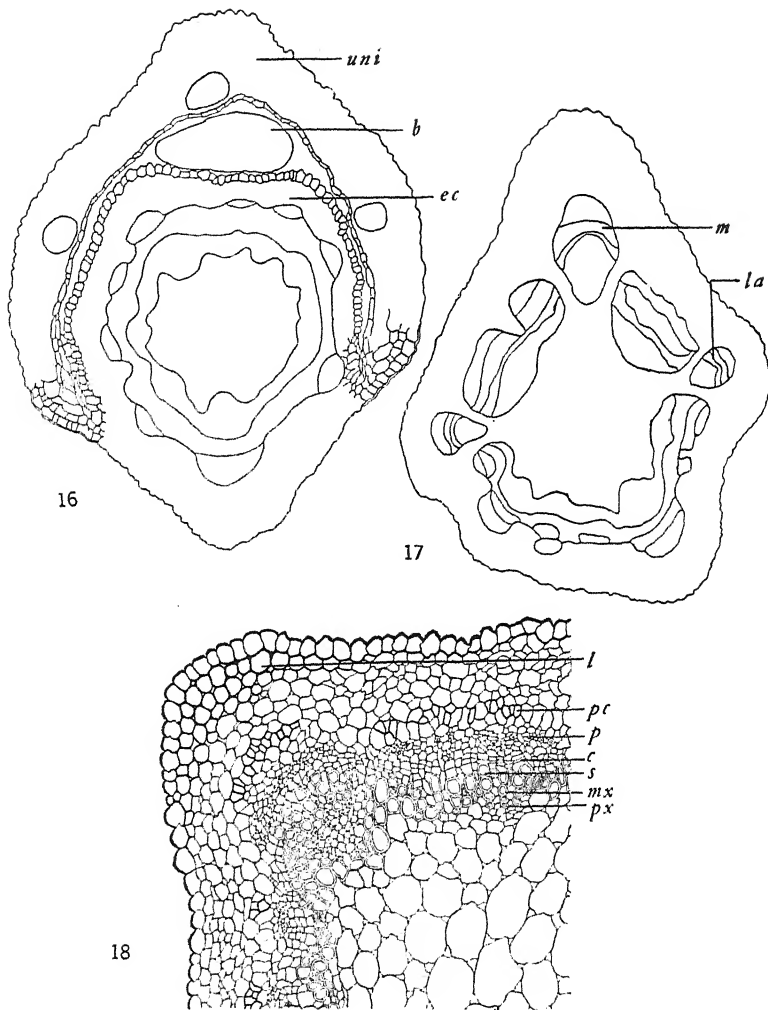
Each trace continues as a single strand through the petiole (figs. 14, 15), and at the base of the blade of the cotyledon the xylem becomes endarch by differentiation of a single rather than of two metaxylem groups and by the further change in position of the protoxylem. A collateral endarch bundle results, in which the protoxylem is directed toward the adaxial surface of the blade. Thus the transition from the exarch to the endarch condition of the xylem is completed in the cotyledons. Branches of the main bundles form numerous vascular strands in the cotyledonary blade.

#### EPICOTYL

In seedlings four to seven days old the epicotyl appears as a mass of meristematic tissue from which the unifoliate leaf is beginning to differentiate. The traces to this leaf appear as small groups of embryonic cells. In seedlings ten to twenty days old a bud may be distinguished in the axil of the unifoliate leaf, and the median and two lateral bundles of the petiole are well differentiated (fig. 16). The stem, which appears circular in cross section in the first internode, becomes square in the third internode, a condition which is typical of the mature stem (figs. 16-18). By the time the seedling is three weeks old a typical dictyostele with endarch collateral bundles is developed in the third internode (fig. 18). In the stem there are prominent fibers in the pericycle over the vascular bundles. The gradual outward differentiation of these fibers may be traced in seedlings ten or more days old from the root through the hypocotyl, where the pericycle becomes more prominent and where many of its cells differentiate as fibers.

#### LEAF TRACES

In a cross section of the upper hypocotyl of a seedling four days old there is noted an isolated xylem element which is in no way connected with the two polar xylem rays of the root which supply the cotyledons (figs. 11, 12). This xylem element differentiates directly above the position of the original third protoxylem point or with



FIGS. 16-18.—Fig. 16, diagram of transverse section of second node from plant three weeks old, showing separation of petiole of unifoliate leaf from axis, with median and lateral bundles differentiated and bud in axil of leaf; fig. 17, diagram of transverse section of second internode, showing divergence from stele of median and lateral traces to first trifoliate leaf; fig. 18, transverse section of third internode from plant three weeks old, showing dictyostele,  $\times 340$ .

equal frequency on the opposite side of the stele. In the young seedling there is no vascular connection between the exarch strands and this developing element; for a short distance above the abortive exarch strand the cells in this region are undifferentiated. In so far as determined, the variation in position of the new xylem element is found in either the type of seedling in which the transition is directly from triarchy to diarchy or the type in which an intermediate tetrarchy occurs. Further study might reveal a relationship between the position of this element and the external anatomy of the seedling.

This xylem is the first indication of an endarch bundle. As the seedling grows, other xylem cells mature adjacent to the first element, phloem matures from the meristematic group of cells adjacent, and these cells together with those differentiating at higher levels form the main endarch collateral bundle which supplies the unifoliate leaf. This trace is therefore continuous from the upper hypocotyl, through the cotyledonary node and through the first internode.

Other bundles appear in the hypocotyl just below the cotyledons by the time the seedlings have reached an age of seven to ten days. They have their origin in the meristematic cylinder of the hypocotyl (fig. 12). The second bundle to differentiate is an endarch collateral trace in the intercotyledonary axis opposite the one just described. This second bundle becomes the median trace to the first trifoliate leaf at the third node. By the time the seedling is two to three weeks old, at least six endarch collateral traces have differentiated in the upper hypocotyl (fig. 12). The two smaller bundles adjacent to the median trace to the third node ultimately differentiate at right angles at the second node as the lateral traces to the petiole of the unifoliate leaf. Thus three traces supply the unifoliate leaf. These continue as three main bundles through the petiole, anastomose at the base of the blade, and then rebranch. The stipular bundles are continuations of the lateral traces.

The two small bundles on either side of the first developed endarch trace ultimately supply the lateral traces to the first trifoliate leaf. As the traces diverge from the main cylinder in the second internode gaps are apparent (fig. 17). Three leaf traces, therefore, are characteristic of this trifoliate leaf. Branches from the lateral traces differentiate in the stipules.

Although the traces to the epicotyl have their origin in the upper hypocotyl, there appears to be no direct continuity of xylem between the primary strands which extend from primary root, through hypocotyl, and into the cotyledons and the xylem of the endarch traces which arise from meristematic tissue in the young seedling. The undifferentiated strands of phloem in the lower hypocotyl of the young seedling seem to be associated in position with these provascular strands which mature into collateral bundles in the upper hypocotyl and in the older seedling. Because of the meristematic nature of these cells, the exact manner of differentiation of phloem between the root and the endarch bundles is difficult to follow; however there seems to be a closer continuity in the differentiation of phloem between root and stem than in the primary xylem. After cambial activity has begun, the secondary tissues of these regions form a more or less continuous system for the movement of water and food materials.

### Conclusion

In *Melilotus alba* the transition from the exarch, triarch, radial arrangement of the vascular tissue of the root is completed in the cotyledons with the formation of endarch collateral traces. The major changes in this transition occur in the upper hypocotyl and in the petioles of the cotyledons. There is a continuous system of primary xylem from root into cotyledonary blades, although one of the original xylem ridges disappears in the lower hypocotyl. The protoxylem, and apparently the metaxylem, of these regions have no direct connection with the primary xylem of the endarch bundles which arise from meristematic tissue in the upper hypocotyl, and which differentiate in the epicotyl as traces to the second and third nodes.

In *Medicago sativa*, WINTER (8) found triarchy, and more rarely tetrarchy, to be characteristic of the primary root, while in the lower hypocotyl tetrarchy invariably occurs. COMPTON (1) noted that in *Melilotus arvensis* triarchy gives place to diarchy in the hypocotyl. The condition in *M. alba* is found to be variable, in that, although triarchy is characteristic of the root, either a diarch or a tetrarch arrangement is found in the lower hypocotyl; however, invariably at a higher level a diarch arrangement occurs. WINTER stated that in

alfalfa "the first vessels in the hypocotyl which are continuous with vessels of the plumule" are produced by cambium. In *M. alba* the collateral endarch traces of the hypocotyl, which continue upward to become the traces to the first plumular leaves, arise from meristematic provascular tissue in the upper hypocotyl in seedlings a few days old. However, the lack of direct connection of the xylem of these collateral bundles with the primary xylem rays of the root and lower hypocotyl in the young seedling resembles the condition found in alfalfa.

The account given by COMPTON (1) of the seedling anatomy of certain species of *Ononis*, *Trifolium*, *Trigonella*, and *Medicago* would indicate that, although variations do occur, there are striking similarities between the mode of transition in these plants and that found in the present investigation on *Melilotus alba*. These similarities, together with the condition described by WINTER in *Medicago sativa*, are indicative of an anatomical relationship between these species of the Trifolieae, and serve as further evidence for a close phylogenetic relationship between the members of this tribe of the Leguminosae.

### Summary

1. The primary root of *Melilotus alba* is characterized by a triarch radial protosteles.
2. The first indication of a change from the triarch exarch arrangement of the xylem of the primary root in the young seedling is the development of diarchy or tetrarchy in the lower hypocotyl.
3. If tetrarchy occurs, it is invariably replaced at a higher level in the hypocotyl by diarchy.
4. The exarch ridge of xylem on the intercotyledonary axis gradually disappears at higher levels in the hypocotyl, and has no direct connection with the primary xylem of cotyledonary or plumular traces.
5. The exarch-endarch transition is continued in the formation of the cotyledonary traces and is completed in the main vascular bundles at the base of the cotyledonary blades.
6. A conspicuous pith differentiates in the hypocotyl, the stele enlarges, the cortex becomes more prominent, and a cutinized epider-

mis is present in this region in the young seedling. An endodermis with Casparian strips is present throughout the greater length of the hypocotyl.

7. A continuous, primary vascular system exists from root, through hypocotyl, into cotyledons.

8. The median vascular bundle which supplies the unifoliate leaf at the second node develops from meristematic tissue in a seedling several days old. The first indication of the differentiation of this collateral endarch bundle is the appearance of a single xylem element in the upper hypocotyl on the intercotyledonary axis, above the disappearing protoxylem of the third ray or on the opposite side.

9. In seedlings seven to ten days old, the median trace to the first trifoliate leaf at the third node, as well as the four bundles which continue upward to form the two lateral traces to the unifoliate leaf and the first trifoliate leaf, respectively, are well differentiated as endarch collateral bundles in the upper hypocotyl. These six primary bundles arise from meristematic tissue and appear to have no direct connection with the primary exarch strands in the lower hypocotyl.

10. Buds arise early in the axils of the cotyledons, the unifoliate leaf, and the first trifoliate leaf. The cotyledonary buds do not ordinarily develop into branches the first season.

11. Cambial activity occurs early in the primary root; fibers are prominent in the secondary xylem and phloem. As a result of cork cambium, which arises in the pericycle, a prominent cork is formed and the cortex of the root disappears.

12. The square stem is characterized by a dictyostele, with collateral endarch bundles.

13. The similarity between the method of transition from root to stem structure in *Melilotus alba* and the types described in *M. arvensis*, *Medicago*, *Trigonella*, *Ononis*, and *Trifolium* indicates a close structural and phylogenetic relationship between these species in the Trifolieae.



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## EMBRYOGENY OF SPECIES OF *PODOCARPUS* OF THE SUBGENUS *STACHYCARPUS*

J. T. BUCHHOLZ

(WITH TWENTY-ONE FIGURES)

### Introduction

The genus *Podocarpus* has been divided into the two subgenera, *Stachycarpus* and *Protopodocarpus*. In 1847, ENDLICHER described the subgenus *Stachycarpus*. The writer has made a study of the embryogeny of three species of the latter subgenus, *Podocarpus spicatus* R. Br., *P. ferrugineus* Don., and *P. usambarensis* Pilger.

The material for this study was obtained from three sources. *Podocarpus spicatus* was collected in New Zealand in 1911 by Dr. E. W. SINNOTT; *P. ferrugineus* was supplied by Mr. H. W. LAWTON, of Wellington, New Zealand; and *Podocarpus usambarensis* Pilger was obtained from Jamaica, where it is grown as an introduced species. While this latter material does not agree in every detail with PILGER'S (6) description of *P. usambarensis*, the large seeds and the leaves exclude it from the only other species that it could be, *P. falcatus*.

SINNOTT (8), in his description and figures of the proembryo of *Podocarpus spicatus*, includes the first sporophytic mitosis, the descent of the four free nuclei to the lower end of the archegonium, the sixteen free nuclei at the pointed end of the archegonium before wall formation, and a proembryo soon after wall formation in which the prosuspensor cells are just beginning to elongate. For *P. ferrugineus*, SINNOTT shows four free nuclei of the proembryo in the upper central part of the archegonium, a stage after their migration to the pointed end of the egg, and a section showing the embryonic tips of two neighboring embryo systems after a period of suspensor elongation. The writer (2) found several additional stages in the early development of the embryo of *P. spicatus*, but the later embryogeny, including the stages of tissue organization and development of cotyledons, remains to be worked out.

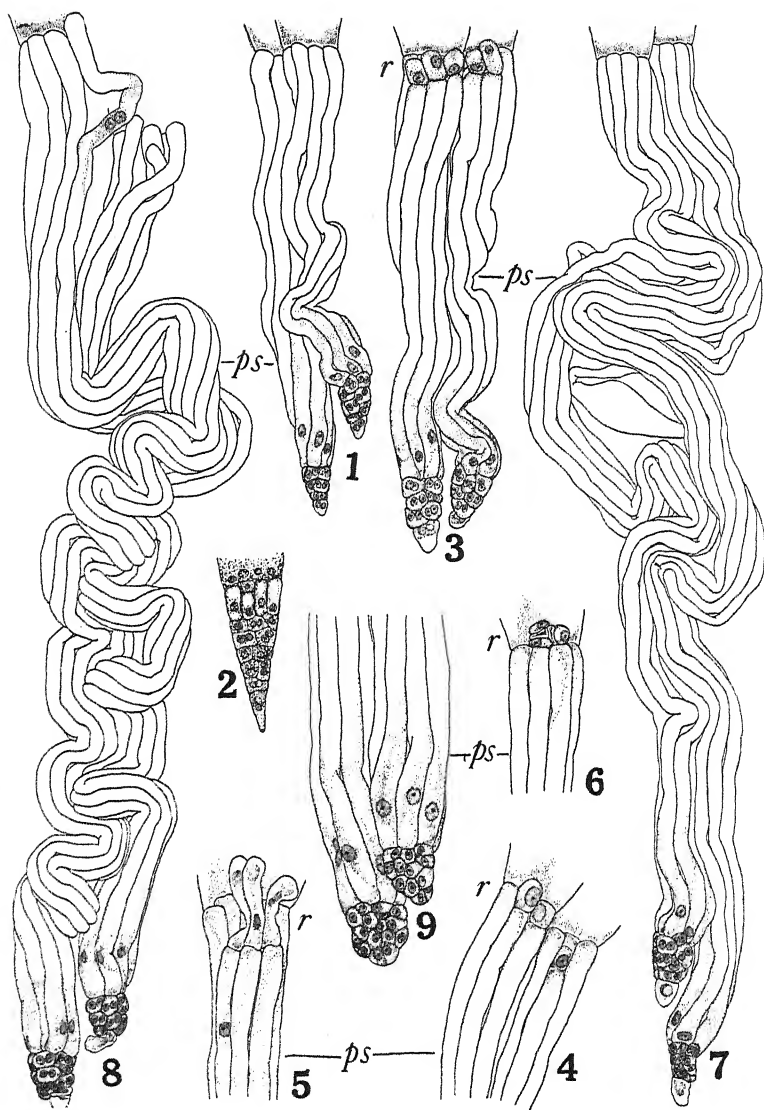
### Investigation

Figure 1 shows two adjacent embryo systems of *Podocarpus spicatus* at an early stage, in which the prosuspensors have slightly elongated. This pair of zygotes shows no rosette cells. There are seven to nine prosuspensor cells, each of which has a nucleus in the lower end of the elongated cell. Each embryo system bears a group of nine to twelve binucleate embryonic cells below the prosuspensors. The writer has stated previously (2) that this binucleate condition of the early embryonic cells seems to be characteristic of the subfamilies Podocarpoideae and Phyllocladoideae. This condition has been found in twelve species. A detailed study of the embryogeny of *Dacrydium cupressinum* (4) has more recently been made.

Figure 2 shows a dissected proembryo of *Podocarpus spicatus*. The structure of the proembryo of *P. ferrugineus*, reported by SINNOTT, was similar to that of *P. spicatus*. In both of these species the proembryo is confined to the lower one-fourth of the slender long-pointed archegonium.

The pair of embryo systems in figure 3 shows several rosette cells and the terminal cap cells which are beginning to disintegrate. Most of the embryo systems of similar age, and nearly all of the older embryos, show no rosette cells (figs. 7, 8). It is possible that this is because the rosette cells collapse and disappear at an early stage of embryo development. The rosette groups of cells shown in figures 4-6 are from zygotes of about the same stage as the zygotes in figures 1 and 3. Figures 5 and 6 show some of the rosette cells beginning to disorganize. The rosette cells are uninucleate, and it is probable that they may be prosuspensor cells which failed to elongate. It is believed that the free nuclei above the prosuspensor in the proembryo of figure 2 soon disorganize and disappear, since they were not found in older material.

The binucleate terminal embryo initials which form the cap cells become aborted and are crushed as the prosuspensor pushes the terminal group of cells downward into the gametophyte. As the prosuspensor elongates, several of the terminal embryo cells may be successively crushed (fig. 8). During the growth of the proembryo the prosuspensor becomes twisted and coiled, and very long. Not all of the elongating prosuspensor cells become the same length. Fewer



FIGS. 1-9.—*Podocarpus spicatus*: figs 3-6, variations in rosette cells; figs. 1, 7, 8, absence of rosette cells (*ps*, prosuspensor; *r*, rosette cells).  $\times 100$ .

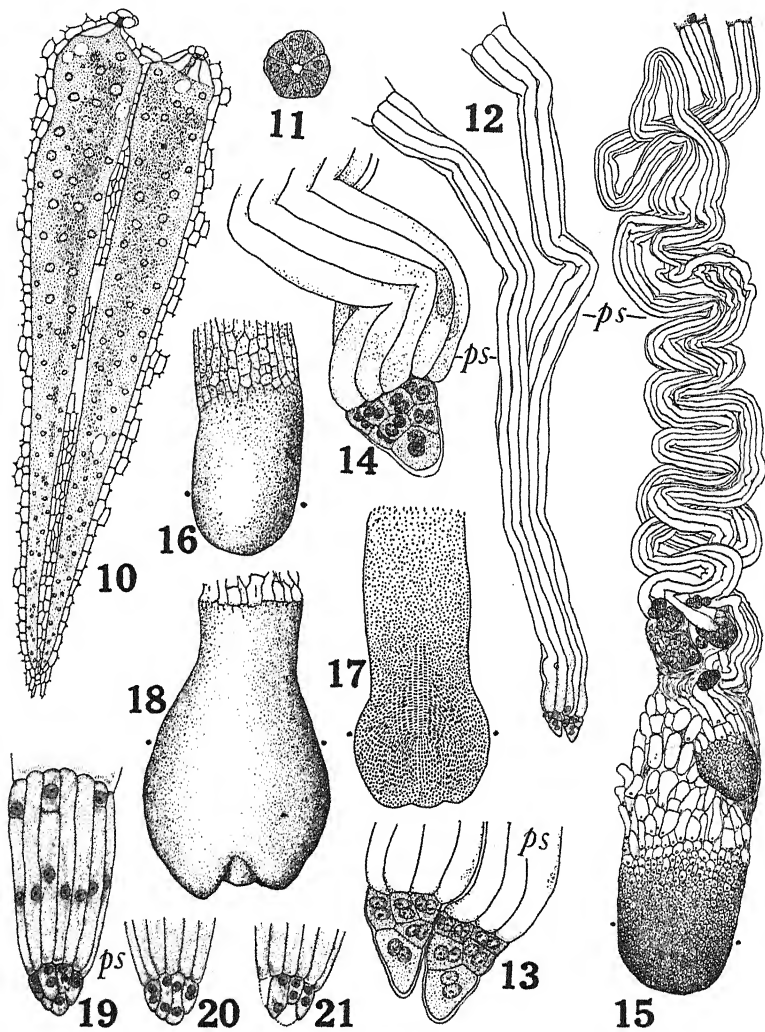
than six or seven cells of an elongated prosuspensor, consisting near the distal end of nine or more cells and lying at the base of the egg, may be joined to the embryonic cells at their proximal end.

As indicated by figures 7 and 8, the embryonic cells remain binucleate for a considerable period during the elongation of the prosuspensor. Ultimately cell plates appear between the two nuclei of each embryo initial or unit, followed by other cell divisions, resulting in a single embryo for each system (fig. 9). Observations have not been made on later stages in the development of the embryo, but it is assumed that the units of each zygote combine to form a single embryo, and that there is no fixed number of these units. One or several of the embryo initials may be broken from the tip of the prosuspensor, and thus an embryo may be formed from only five or six of these initials. On the other hand, there may be as many as ten or more units which form the cylindrical body of the later embryo.

Both *P. spicatus* and *P. ferrugineus* have long-pointed archegonia. Figure 10, from a dissection, shows two adjacent archegonia of the latter species at the time of fertilization. There are many protein granules and vacuoles scattered throughout the cytoplasm of the egg and it is difficult to identify the nuclei. The two archegonia of figure 10 are slightly flattened against each other, so that they appear somewhat narrower in the figure than when viewed from a plane at right angles.

Figure 12 shows the prosuspenders of two neighboring systems of *P. ferrugineus* after they have elongated. Figure 13 is a view of the lower part of figure 12. There are six or seven binucleate units in the embryo at the left, and nine in the one at the right. Figure 14 belongs to another embryo system in which there are ten binucleate cells and the prosuspensor has become more elongated and excessively twisted. These figures show that *P. ferrugineus* has binucleate embryo initials, but unlike *P. spicatus*, the terminal cell in the few specimens examined appears to persist and may contribute to the embryo. Figure 15 shows a considerably older stage of development than figure 14. This material was obtained from a dry seed in which there were more embryos than the number usually found in two zygotes, so in this instance there must have been a cleavage in the group of binucleate units in one of the two zygotes whose prosus-

pensors are still visible above. Evidently here cleavage polyembryony is the exception and simple polyembryony is the usual condition.



FIGS. 10-21.—Figs. 10-18, *Podocarpus ferrugineus*; figs. 19-21, *P. usambarensis*. Fig. 11, polar view of neck cells of an archegonium. Figs. 10, 12,  $\times 55$ ; figs. 11, 13, 14,  $\times 210$ ; figs. 15-18,  $\times 40$ ; figs. 19-21,  $\times 100$ .

From some dry seeds of *P. ferrugineus* there was dissected a suspensor system over 8 mm. in length. The embryo, although broken

off during dissection, was too small to be identified. The seeds of this species seem to require a long rest period before germinating, possibly because the embryo is very small and must undergo considerable development before the seed is ready for germination. Mr. LAWTON, the collector of the seeds, stated that they may require months to germinate. The cause of the delayed germination, however, should be more thoroughly investigated in the region where they grow. A study should be made of the stages of development of the embryo both before and after the seeds are shed. Only a few of the embryos in the several dozen seeds that were dissected showed cotyledons beginning to develop, and in only one of these were the cotyledons longer than those seen in figure 18. The embryo of *P. ferrugineus* in figure 18 is, with two exceptions, in the oldest stage of development of any that could be found in the dry seeds. All of these embryos are immature when compared with the embryos found in ripe seeds of most conifers.

In figure 17 the plerome of the root tip is seen near the center of the spherical part of the embryonic tissue. The shading here approximately indicates the orientation of the cells. The tissue on the side toward the secondary suspensor is the periblem, root cap, and suspensor combined. The upper cells of this cylindrical mass of embryo cells elongate successively to add to the massive secondary suspensor. The cells nearest the tip and at the sides of the plerome constitute the periblem. The root cap region is composed of an undifferentiated mass of periblem and calyptragen cells, similar to those found in *Cedrus*, which BUCHHOLZ and OLD (5) have called calyptrorperiblem.

The plerome of the root tip may be recognized even in the optical sections of cleared whole mounts. In the terminal embryo of figure 15 the tip of the pleromic arc of the root was seen at the level between the two black dots placed at each side of this figure. Black dots also mark the position of this structure in the embryos of figures 16-18. Thus only a small part of the cylindrical embryonic cell mass forms the embryo. It is probable that the entire later embryo is derived from a few, or only one, of the earlier binucleate units, the others giving rise to cells which ultimately contribute to the suspensor and calyptrorperiblem.

The primordia of the stem tip and cotyledons are the next structures to develop. The meristem of the stem tip is visible between the two cotyledons in figure 18.

From a study of the embryos of *P. ferrugineus*, dissected from soaked seeds, it appears that there is usually no cleavage polyembryony and therefore no primary suspensor is formed. There is no single-celled structure such as the primary suspensors which may be seen in *Sciadopitys* (3) or *Biota* (2). The prosuspensor elongates greatly, as shown in figure 15. On the end of this, the binucleate group of cells, such as those shown in figures 13 and 14, usually later combines to form a cylindrical mass composed of several hundred cells. When the prosuspensor has reached its maximum length, a massive rope of many embryonal tubes elongates to form the secondary suspensor.

It is possible that the development of the embryo from the binucleate initial cells will be the same in *P. spicatus* (fig. 9) as in *P. ferrugineus*. The embryo system in *P. spicatus* probably does not normally undergo cleavage, and there is no primary suspensor.

Only one early stage in the embryogeny of *P. usambarensis* was found, but this is a very important one, since it shows the organization of the late proembryo (figs. 19-21). Figures 20 and 21 were drawn from the same embryo in successively deeper planes of focus; a total of nine binucleate embryo initials were seen. The embryo initials have become unequal in size, and all of them are oriented in a single tier with their long axes parallel to the axis of the prosuspensor. The cells of the prosuspensor are eighteen to twenty in number and their single nuclei are almost centrally located. Several rosette cells were found above the prosuspensor cells, and as usual there were many free nuclei above the rosette cells which were lost in dissection.

As shown in figures 19-21, the prosuspensor is very massive, consisting at first of twenty or more cells. Dissection of the later stages shows that the embryo of *P. usambarensis* develops a long prosuspensor, which is spirally and irregularly coiled in the cavity between the small terminal embryo and the micropyle. No evidence of cleavage polyembryony was observed in about a dozen dissections of the specimens which were examined. The prosuspensors were greatly



elongated. In the later embryo a distinct stem tip meristem, similar to that of *P. ferrugineus*, was recognizable, but the embryos of mature seeds were well developed.

There is no apical initial cell in the embryo of *P. ferrugineus* or *P. spicatus*, and furthermore there seems to be no apical cell growth in the embryonic cell masses at any stage of their development.

PILGER (7) noted the length of the suspensor in *P. amarus*, a species closely related to *P. usambarensis*, which he dissected from a mature seed. This suspensor system, which was 30 mm. long, was spirally coiled in a cavity beginning at the micropyle and was terminated by an immature embryo in which the cotyledons had not begun to develop.

### Discussion

The material for comparison of embryo development was not abundant, but the writer was fortunate in having stages of the three species which corresponded closely enough to permit critical comparisons, and to make what seems to be a reasonably accurate reconstruction of the organization of the late proembryo. The range in diversity of formation and development of the embryo, within the restricted group studied, is pronounced, and calls for an explanation of the causes of the diversity. The differences in embryogeny are greater than those found among the genera of other families, or even between other families of the conifers.

A study of the morphology and taxonomy of the three species of *Stachycarpus* seems to indicate that the species representing the two extremes of structural features are *P. spicatus* and *P. usambarensis*; the intermediate form is *P. ferrugineus*.

This subgenus apparently presents a complete series of evolutionary steps in its external morphology. The features of the embryogeny which are known sufficiently to permit comparison show corresponding differences, but there is a question as to whether the internal changes were at all synchronized with the changes in external morphology. Apparently a massive prosuspensor (which develops into a structure of great length, having many twists and coils) and the organization of eight to twelve binucleate embryo units are features common to all three genera, and may therefore represent the

most conservative feature. *P. spicatus* has the binucleate embryo units organized into four, five, or more tiers in the early embryo; *P. ferrugineus*, into three or four; and *P. usambarensis* has them in a single tier. Thus these details in the arrangement of the binucleate units of the embryo initials are more variable features which are of lesser importance phyletically, but significant ontogenetically. *P. usambarensis* has a much larger number of prosuspensor cells than *P. spicatus* and *P. ferrugineus*, while the deciduous cap cells found in the embryos of *P. spicatus* are not present in the other two species.

Apparently an evolutionary series is suggested by these differences in arrangement of binucleate embryo units. Either the many tiers represent an advanced condition and the single tier a primitive condition, or the reverse.

These alternatives must be kept in mind as the embryogeny of other podocarps is studied, for in *Dacrydium* there are only two tiers of binucleate cells, or at most three, and in *P. totara* (8) there is only a single tier, represented by a single terminal binucleate cell.

There is also the possibility that these modifications in the organization of the embryo are occasioned by the pronounced differences in the shapes of the archegonia. The archegonia of *P. usambarensis* are numerous, at least four or five in number, and not long pointed; those of *P. spicatus* and *P. ferrugineus* are usually two in number, and extremely narrow and long pointed.

If it is assumed that the changes in internal embryogeny kept pace with evolutionary changes in the external morphology, then it may be expected that the type represented by the large seeded *P. usambarensis* is the most advanced member of the series. Returning to a consideration of the external morphology, it is evident that *P. spicatus* and *P. andinus* (6) have a definite ovulate cone axis bearing six to nine or more bracted ovules in spiral succession, the entire spicate arrangement constituting a loose ovulate cone. In *P. ferrugineus* there is usually only a single ovule, which matures on the end of a stalk representing the cone axis.

In both these cases, as SINNOTT has shown, the epimatium, or the morphological equivalent of the cone scale, is fused with and surrounds the ovule so that the ovules appear to have an extra outer integument. These members are borne in the axis of a bract with

vascular connections comparable with those of the bract and scale of the Pinaceae. In *P. usambarensis* as in *P. ferrugineus*, the number of ovules has been reduced to one, which in *P. usambarensis* becomes very large and is borne on the end of a cone axis having many nodes. Thus *P. spicatus* is the least specialized and *P. usambarensis*, with its very large seed, is the most specialized in external morphology—obviously an evolutionary series in which a single stalked ovule has been evolved from a loose spicate strobilus. If the changes in internal embryogeny have kept pace with, and run parallel to, the evolution in the external anatomy, then the embryo of *P. spicatus* (and the unknown embryogeny of *P. andinus*) should present the most primitive condition. On the other hand, we should also consider the cause for the changes in the shape of the archegonia. These changes doubtless affected the organization of the embryo. In their more primitive condition the archegonia were probably not narrow and long pointed, but of the oval type usually found in conifers, a condition which exists in the archegonial group of *P. usambarensis*. We may therefore consider that the pronounced differences in the organization of the late proembryos of these three species have in some way been connected with the change in shape of the archegonia, so that the binucleate embryonic units which are found in a single tier of *P. usambarensis* have become arranged in three or more tiers in the narrowed archegonium of the other species. Thus one may see the possibility of a causal relationship in the modification of the embryogeny from the original type. The embryos of *P. spicatus* and *P. ferrugineus* may therefore represent the specialized condition, and *P. usambarensis* the more primitive condition. These suggestions are provisional, and definite conclusions cannot be reached until more facts concerning the embryogeny of these and other podocarps are available.

The embryo of *P. spicatus* has been compared with that of *Cephalotaxus* by the writer (1) and others in regard to the existence of a deciduous cap cell at the distal end of the embryo. The writer later pointed out that these cap cells in *Podocarpus* are always binucleate, while those of *Cephalotaxus*, as well as its functional embryonic cells, are uninucleate structures. The apparent similarity is

therefore only an interesting parallelism in evolution. Similar cap cells occur also in the embryos of *Sciadopitys* (3). Likewise all the rosette cells of these podocarps abort and disappear early, while those of *Cephalotaxus* usually give rise to rosette embryos.

Another difference between the embryo of *Cephalotaxus* and these embryos of podocarps is in the time of appearance of the stem tip of the latter embryo. The meristem of the stem tip is delayed in *Cephalotaxus* and is not present as an emergent structure in an embryo at the stage found in the mature seed. In *P. ferrugineus* the stem tip is present in the embryo and is recognizable at least as early as the cotyledons. We may expect the embryo of *P. spicatus* to agree with this. Similarly, in the embryo of *P. usambarensis* the meristematic dome of the stem tip is recognizable long before the embryo completes its development within the seed. Thus when the early and late embryogeny of *Cephalotaxus* and these podocarps are compared, little similarity is found between them.

### Summary

1. Pronounced differences are found in the appearance and organization of the early embryos of *Podocarpus spicatus*, *P. ferrugineus*, and *P. usambarensis*, which belong to the subgenus *Stachycarpus*.

2. All three species have seven to twelve binucleate embryonic cell units. These units give rise to groups of cells which usually combined to form a single embryo; thus simple polyembryony prevails within this group.

3. The embryogeny of these three species suggests a sequence of evolutionary steps which appears to be opposite to the sequence suggested by study of the external morphology of the seed cone—the reduction series from a loose strobilus to a stalked ovule. Causal morphology may explain the modifications in the development of the embryo as due to changes in the shapes of the archegonia.

4. No apical initial cell could be observed at any stage in the development of the embryo of any of the species.

5. The resemblances between the embryos of *Cephalotaxus* and those of certain podocarps are superficial and do not suggest close phylogenetic connections.

6. In *P. ferrugineus* and *P. usambarensis* the stem tip meristems are organized and recognizable between the cotyledons of the embryos before the seed is mature.

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## SECONDARY ROOT HAIRS

M. ELIZABETH PINKERTON

(WITH EIGHT FIGURES)

In certain species of *Tradescantia* heavy-walled root hairs which form a persistent white or brown tomentum upon dried specimens have been noted. It was the purpose of this research to investigate the anatomical features of the roots of the Commelinaceae in particular, as regards the root hairs, and to try to determine the function of the latter.

### Literature

As early as 1877 DE BARY (2) distinguished two types of root hairs, transitory "Safthaaren" with contents, and persistent dead, air-filled "Deckenhaaren." SCHWARZ (13) thought that their delicate nature facilitated absorption. The hairs soon became brown, disorganized completely, and at the same time the epidermis died. Their death, however, occurred before periderm formation, was unrelated to it, and was inevitable whether the root was in air, soil, or water. Later he recorded observations of turgid hairs in maize at a distance of 20 cm. from the tip, in apparent contradiction to his previous observations. GRAVIS (4) noted that in *Tradescantia virginica* the "assise pilifere" became suberized early and persisted at the surface of the older roots as empty cells with brown walls and of rather withered structure. It was assumed by KROEMER (8) that there was a secondary state of the "Aufzellen" achieved with the cutinization of cell walls and loss of protoplasm. The occurrence of hairs with thickened walls in a number of widely separated families and their special abundance in xerophytes was mentioned by HESSE (6). He assumed that absorption through them would be impossible owing to the heavy walls, but that an anchorage function was likely.

LEAVITT (9) came to the general conclusion that root hairs had two types of origin. In the simple type any cell of the epidermis might become piliferous, with no apparent predetermination; in the other there was differentiation under the root cap into small wedge-

shaped densely staining cells, which he called "trichoblasts," and larger, lightly staining cells. The former elongated little but usually became papillate, whereas the intervening cells did increase in length but not laterally. Owing to the horizontal extension from the wedge-shaped bases of the trichoblastic cells, the atrichoblastic epidermal cells might be sloughed off. In some plants having the simple origin of epidermal hairs he observed that there was a differentiation of the exodermis into "regular" and "transfusion" cells. The latter, swelling tangentially, forced aside the epidermis and became functionally absorptive. Chemical tests indicated that these transfusion cells had cellulose walls whereas the other hypodermal cells were cutinized. Roots having the trichoblastic mechanism lacked the transfusion apparatus, and phylogenetically the two conditions were generally segregated. According to LEAVITT, the dicotyledons and some monocotyledons (including the Liliiflorae, Spadiflorae, Orchidaceae) have simple epidermal hairs replaced by a transfusion-cell piliferous layer from the cortex; whereas the majority of the monocotyledons have the predetermined trichoblastic layer which is morphologically a cortical member.

HABERLANDT (5) quoted KROEMER to the effect that sometimes the walls of persistent hairs undergo lignification or become impregnated with protective substances of unknown composition. He mentioned specifically long-lived hairs on two epiphytic ferns, *Drymoglossum nummularifolium* and *D. piloselloides*. WATSON (15) merely described the occurrence of root hairs on old roots of *Helianthus rigidus*.

The first attempt to explain the persistence of root hairs upon an anatomical basis was made by JEFFREY and TORREY (7). They maintained that there was a correlation between the presence of persistent hairs and the absence of secondary growth of the root in *Aster*, and that this condition led to a higher absorptive efficiency. McDUGALL (10) investigated the prevalence of thick-walled root hairs in the Caesalpiniodae, and stated that the roots appeared like ectotrophic mycorrhiza, and that the individual root hairs were dark brown and did not shrivel. In this subfamily of Leguminosae they are a constant feature even at depths of 4 feet, and seem normally to last as long as the epidermis. It was impossible to determine the

nature of the contents, if any, because of the dense walls of the hairs whose chemistry was not investigated. The suggestion was offered that the persistence of root hairs, as well as of thorns, might be an archaic condition of a xerophytic type now adapted to various habitats.

WHITAKER (16) investigated a number of genera of the Compositae as well as certain of the Leguminosae and came to the general conclusion that there is a definite correlation between a permanent hirsute condition and lack of secondary thickening. It was intimated that such hairs might be functional, since she stipulated that the radial arrangement of the primary root stele was adapted to absorption whereas the concentric disposal of the secondary one was not. Another casual statement was of interest; namely, that the exodermis in contrast to the endodermis was probably a fairly new and rather inconstant feature of root organization, but occurred constantly in the species studied by her. It is the opinion of the present writer that this exodermis is definitely associated with the production of secondary hairs as will be proposed in a subsequent paragraph.

A brief comment upon WHITAKER's paper by POPESCO (12) may be translated: "WHITAKER describes some plants which never produce secondary tissues in their roots and which absorb just the same; she names them *apilose*." Apparently POPESCO had not grasped the meaning of her term which signified simply a condition where root hairs were lacking. On the persistent feature he made no comment, nor did he mention it in his own research except indirectly that in *Sambucus nigra* there were alternating zones of young and old hairs, proving that the hairs were not absorptive but had "quite another function" which he did not discuss. ARBER (1) has stressed the peculiarity of the typical monocotyledonous root associated with the lack of secondary thickening and absence of deep-seated periderm. She states that the root hairs were surrounded by jacket cells and contained delicate simple or branched rods of highly refractive substances at their bases. It is not clear what she meant by the former, but perhaps they would correspond to the atrichoblastic cells of LEAVITT. She also defines the exodermis as a generally cutinized layer broken by passage cells and occurring below the piliferous one.



MILLER (11) states: "The length of life of root hairs depends on the species of the plant and upon the moisture conditions of the soil. Root hairs of some species live for only a few days or weeks, while those of others exist for several years, although they do not function in absorption for a period that long."

Thus it appears that although thick-walled root hairs have been noted for some time, the general assumption has been that they are merely persistent primary hairs whose function has not been satisfactorily explained.

### Material and methods

The following served as research material:

COMMELINACEAE.—*Tradescantia edwardsiana* Tharp, *T. geniculata* Jacq., *T. gigantea* Rose, *T. hirsuticaulis* Small, *T. hirsutiflora* Bush, *T. occidentalis* Smyth, *T. paludosa* Anderson and Woodson, *T. reverchoni* Bush, *Setcreasea brevifolia* (Torr.) Rose, *Palisota barteri* Hook., *P. bracteosa* C. B. Clarke, *P. mannii* C. B. Clarke, *Dichorisandra thyrsoflora* Mikan, *Commelina coelestis* Willd., *C. communis* L., *Tinantia fugax* Scheidw., *Rhoeo discolor* Hance, *Zebrina pendula* Schnizl.

OTHER MONOCOTYLEDONS.—*Philodendron cordatum* Kunth, *Asparagus officinalis* L., *Zea mays* L.

The root material was studied in regard to the superficial aspect, the internal anatomy, and the physiology.

### SUPERFICIAL ASPECT

A number of species were examined from herbarium specimens with regard to color, extent, approximate length, and tenacity of the root hairs. Small cuttings of *Rhoeo discolor* and *Dichorisandra thyrsoflora* were grown in a water chamber fitted on the stage of a microscope, so that a fresh solution of tap water was kept flowing continuously (apparatus copied after FARR, 3). Cuttings of mature *Rhoeo discolor* had been taken from the soil and immersed in water to produce usable rootlets within a week's time. The cuttings of *Dichorisandra thyrsoflora* were obtained from plants being cultivated in water.

## ANATOMY

The specimens were examined in the living state, both superficially and as freehand sections, and also as preserved material. Most of the latter was killed in chrom-acetic acid or Jeffrey's picric acid-corrosive sublimate reagent, dehydrated through Zirkle's butyl alcohol series, imbedded in paraffin and cut at 10  $\mu$ . Celloidin was used as an alternative matrix and proved superior for keeping the hairs intact. Safranin and light-green was the most effective stain combination for paraffin sections, whereas iron-alum haematoxylin served for the celloidin. In order to determine the nature of the root hair walls, microchemical tests were made with chloriodide of zinc as a test for cutin, and KOH plus the latter for suberin. Various stains were also used, especially safranin, Congo red, and Magdala red. The tests employed by SCOTT (14) were tried also.

## PHYSIOLOGY

A cutting of *Setcreasea brevifolia* which had been growing in tap water for a number of days and had produced a root of several centimeters was fitted into the water chamber. This was attached to a 5 liter flask from which a solution of methylene blue (1:1,000,000) was suctioned off. The rate of flow was adjusted by a stopcock so that the solution was exhausted in about 12 hours; it was then re-employed for the length of the experiment, 36 hours. The whole apparatus was kept in a dark room with an even temperature and good ventilation. The purpose of this procedure was to attempt a vital staining in order to note contents and activities of the hairs.

An attempt was made to follow POPESCO's (12) method with iron sulphate and potassium ferrocyanide. His description was indefinite as to whether ferric or ferrous sulphate was utilized and as to the amounts and the time. Dilutions of 1:10,000 ferrous sulphate employed overnight gave the best results in the present experiment. Cuttings of *Commelina communis* grown in tap water for several days were immersed in the ferrous sulphate solution in three series: those normal and intact; those incised in the zone of older hairs and immersed directly; and those incised as above and sealed with melted vaseline.

## Results

### SUPERFICIAL ASPECT

Externally the majority of the Commelinaceae have roots which are slender but somewhat succulent, and more or less completely and luxuriantly hirsute from near the tip to approximately the top. Often the lower centimeter or so is hyaline and the rest of a dark brown or occasionally white tomentum. The former condition is typical of *Rhoeo discolor*, the primary experimental plant. The species of *Palisota* and *Dichorisandra* show a similar condition, the delimitation of white and brown hairs being conspicuous even in the soil. The roots of *Tradescantia reverchonii* are very hirsute. These plants are of tropical habitat but widely planted in greenhouses. *T. hirsuticaulis* has peculiar little drop roots thickened at the distal ends from which protrude tiny rootlets. This type of root, rather common in the monocotyledons, is especially associated with bulbous plants and has a contractile function (1). *T. edwardsiana*, *T. gigantea*, and *T. occidentalis*, found in moist fertile soils of the Mississippi Valley, have essentially the same type of piliferous layer. *T. paludosa*, a lesser plant with smaller roots, grows in drier regions and has longer hairs associated with a slightly different anatomical structure. *Zebrina pendula* shows a minimum amount of hirsuteness even in water-cultivated specimens, and the roots are small for experimental purposes.

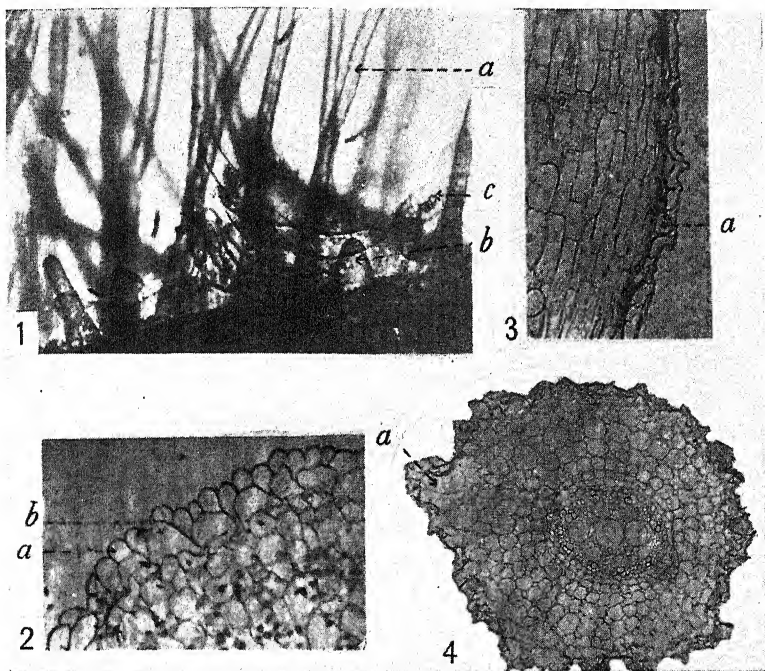
Cuttings of *Commelina communis* grown in water develop white hairs under the surface and brown ones on all exposed parts above it, except for the uppermost centimeter or so which is glabrous.

The roots of *Philodendron cordatum* have a brown tomentum externally not unlike most of the species of *Tradescantia*. *Asparagus officinalis* is very hirsute and *Zea mays* to a lesser degree.

The use of external root hair features as delimiting characters in taxonomy seems feasible, since there is a constancy in this regard in plants otherwise showing close relationships.

Microscopic observations of a specimen of *Rhoeo discolor* growing in the water chamber first revealed the origin of the brown hairs. Microscopically there seems to be an abrupt change from hyaline to brown hairs, but magnification portrays a narrow transition zone

where among the long slender transparent hairs the short russet thicker ones are noticeable (fig. 1). The latter are of secondary origin, that is, they come from the exodermis and supersede the primary hairs. They arise usually several centimeters from the tip in the region of older epidermal hairs. The latter, together with the cres-



FIGS. 1-4.\*—Fig. 1, origin of secondary hairs in *Rhoeo discolor*,  $\times 450$ : a, primary hair; b, secondary hair; c, displaced epidermal (atrichoblastic) cell. Fig. 2, enlarging hypodermis in *Tradescantia paludosa*,  $\times 100$ : a, epidermal cell; b, hypodermal cell pushing aside epidermis. Fig. 3, longitudinal section showing origin of secondary hairs in *Philodendron cordatum*,  $\times 100$ : a, hypodermal cell becoming piliferous. Fig. 4, cross section of mature root of *Rhoeo discolor*,  $\times 30$ : a, origin of secondary hair.

\* All drawings made with an Abbé camera ludica at  $\times 700$ . Reduction approximately two and one-half times.

cent atrichoblastic cells (9), are pushed aside as the new ones emerge from the exodermis. From the outset they are sturdier than their predecessors and their heavy walls effectively mask any contents. Seedlings of *Tinantia fugax*, *Commelina coelestis*, and *Tradescantia*

*geniculata* germinated on filter paper revealed the presence of secondary hairs at a young stage when the rootlets were only several centimeters long.

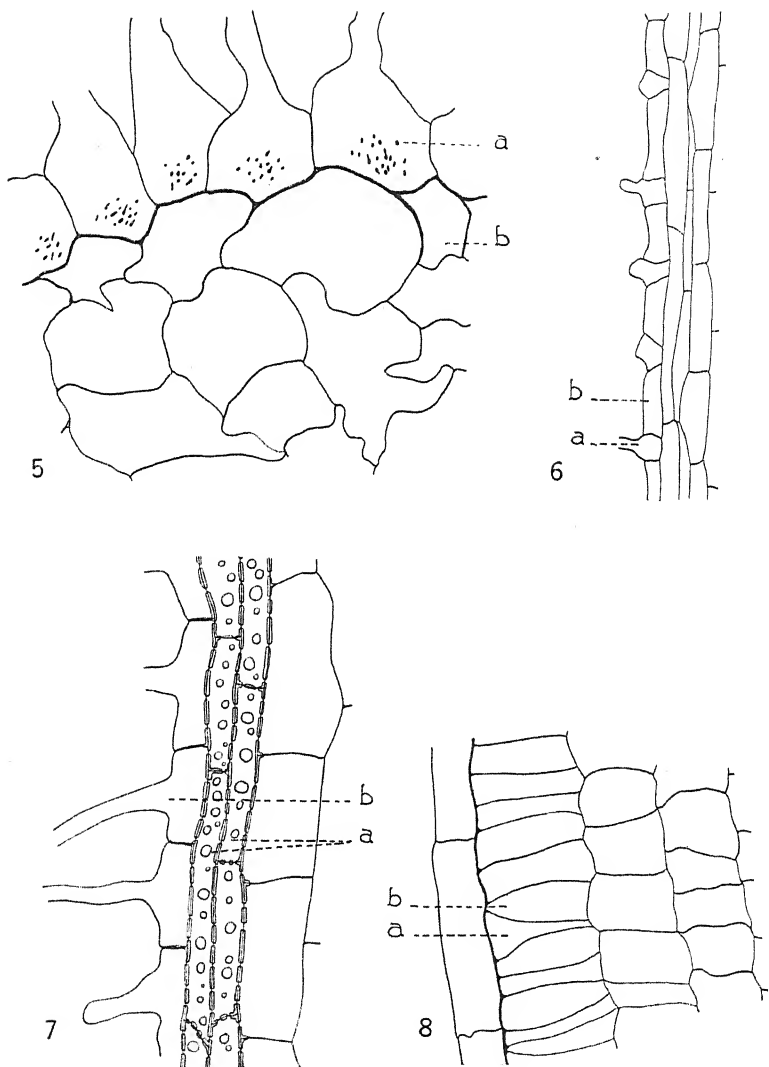
No attempt was made to correlate data from the dicotyledonous roots, but those cursorily examined from a number of garden plants showed only epidermal hairs. Seedlings of lettuce germinated in a moist chamber indicate, however, that new hairs may arise at various indiscriminate places in the piliferous layer, which is contrary to the general view that they are formed only in acropetal succession.

#### ANATOMY

Most of the roots of the Commelinaceae display the following structural features: a relatively small stele, usually polyarch, with several large lacunae in the pith and with no secondary growth; a thin-walled endodermis, often detectable in cross section only by Casparian strips; a prominent turgid cortex whose cells are amply supplied with plastids and starch grains and surrounded by large air spaces; an exodermis of one to several layers with walls thickened on the three outer sides, excepting the passage cells; and a piliferous layer (fig. 2). In the oldest parts of the roots of *Commelina communis* there are no hairs. Internally the endodermis has gone to the tertiary stage (become completely suberized), so there is no interchange of material between cortex and stele. The roots of *Philodendron cordatum* are similar to the preceding, except for the distinction of mucilage canals and isolated tracheids in the cortex (fig. 3). *Asparagus officinalis* has several layers of cylindrical exodermal cells (fig. 7).

Cell contents, including nuclei, are visible in stained secondary hairs of fresh material at a distance of several centimeters from the tip. The walls also appear irregularly thickened internally. The heavy-walled hairs give the test for cutin. In *Dichorisandra thyrsiflora* the inner walls of the piliferous layer are lined with tiny granules which stain red with safranin (fig. 5). This phenomenon was noted also by ARBER (1).

The trichoblastic-atrichoblastic condition is well shown in *Palisota barteri* (fig. 6). In *Tradescantia gigantea* the exodermis is delimited at an early age. The conspicuous exodermal layer of large



FIGS. 5-8.—Fig. 5, *Dichorisandra thyrsiflora*: a, refractive granules; b, cutinized hypodermal cell. Fig. 6, *Palisota barteri*: a, trichoblast; b, atrichoblast. Fig. 7, thickened pitted hypodermal cells in *Asparagus officinalis*: a, hypodermal cell; b, epidermal cell. Fig. 8, differentiation under root cap in *Tradescantia gigantea*: a, trichoblast; b, atrichoblast.

protruding cells in *T. paludosa* shows lateral expansions (fig. 2). Further development of the cortical hair initials appears in *Rhoeo discolor* and in *Philodendron cordatum* (fig. 4).

#### PHYSIOLOGY

The few experiments tried with dyes were not convincing. After 30 hours in weak methylene blue solution, cuttings of *Setcreasea brevifolia* exhibited root tips colored blue for a distance of half a centimeter. Sections showed staining mainly in the stele, although there was some in the cortex and an indeterminate condition in the brown hairs.

Results following POPESCO's (12) technique were unsatisfactory also; the stele and the piliferous layer were stained but not the cortex.

The brown hairs of *Rhoeo discolor* themselves have a fuzzy appearance owing to the small particles of undetermined substances clinging thereto, which may be formed by chemical precipitation and indicate absorption. Their walls are thickened and not gelatinous like those of the primary hairs.

#### Discussion

The conclusion of JEFFREY and TORREY (7) and WHITAKER (16), that the occurrence of "persistent hairs" is associated with the lack of secondary growth, applies to those monocotyledons where secondary thickening is known only infrequently. But the fact that the hairs most certainly arise secondarily and are obviously of physical structure different from the primary ones is puzzling. Since their walls are thickened, as well as those of the cells lying immediately underneath, absorptive ability is made doubtful in spite of passage cells in the exodermis. On the other hand the observed contents (including nuclei, external adherent particles), the turgid cortex, and primary endodermis, suggest that absorption may be possible.

Anchorage may be another function of secondary root hairs, as several investigators have suggested. The facts that these plants have a comparatively shallow root system and often grow in open soil, as well as that there is a tenacious adherence of soil particles,

further this hypothesis. In one case, *Tradescantia hirsuticaulis*, the epidermal layer is obviously specialized for contraction.

If, as WHITAKER (16) stipulates, the exodermis is a rather new feature of root organization, its constant presence in these fundamental groups of monocotyledons suggests a relatively late origin, concomitant with that of the herbaceous Compositae and Ranunculaceae, at a time when perhaps environmental conditions were such that an expansive surface of hairs was for some reason desirable.

LEAVITT'S (9) exclusive segregation of the monocotyledons with trichoblastic layers and dicotyledons with transfusion layers does not seem justified. In certain representatives of the former, the writer finds that the original hairs are of trichoblastic nature and predetermined in the young part of the root under the root cap (fig. 8). The secondary hairs, in the same, arise from exodermal cells (transfusion layer); and, as shown in *Palisota barteri*, apparently the trichoblastic condition repeats itself (fig. 6).

### Summary

In the members of the monocotyledons investigated there exists a so-called "persistent" hirsuteness of practically the whole root system. The constituent cutinized hairs have been found to be of secondary cortical origin, superseding the primary epidermal ones. It seems logical that this condition may be associated with the lack of secondary thickening, but the function has not been determined.

Appreciation is due DR. R. E. WOODSON, JR., who suggested this problem and assisted its progress, and DR. G. T. MOORE for the use of the facilities of the Missouri Botanical Garden.

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# MICROSPOROGENESIS AND CYTOKINESIS IN ASIMINA TRILOBA

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 474

JOHN FLOWERS LOCKE

(WITH PLATE I)

## Introduction

The North American pawpaw, *Asimina triloba* (L.) Dunal, occurs throughout nearly the whole of the eastern half of the United States, exclusive of the New England states.

Little cytological work has been reported on the two genera of the Anonaceae, *Asimina* and *Anona*, which occur in the United States. VOIGT (8) investigated the structure and development of the peculiarly ruminated endosperm in *Uvaria lowii*, and stated that the other members of the family show no marked differences in either structure or development. NICOLosi-RONCATI (4, 5) reported on the formation of the endosperm and the structure of the ovule in *Anona cherimolia*. HERMS (1) described a few stages in the early development of the megagametophyte in *Asimina triloba*. SAMUELS-SON (7) reported that microspore formation in *Aristolochia clematitis* is by successive constriction, that the same type of constriction occurs in *Asimina*, and discussed the systematic significance of such a type of microspore formation.

Since none of these reports deal with meiosis and the number of chromosomes, a study has been made of nuclear behavior during meiosis in *Asimina triloba*. On examining the material of *Asimina*, a peculiar type of cytokinesis was noticed; and since the heterotypic prophase of the meiotic divisions showed little of significance, this investigation has concerned itself mainly with the type of cytokinesis occurring in the divisions of the pollen mother cells in the formation of microspores.

## Materials and methods

Material of *Asimina* was collected in the spring of 1931 near Mississippi State College and in 1933 and 1934 in the region of Smith,

Indiana. Most of the material was killed in the field. The first step in the preparation of the material was to remove the sepals and petals from the floral buds. Good penetration was obtained when the buds were dropped in Carnoy's solution for a few seconds before being placed in the fixing solution. For the earlier stages good results were obtained by the use of a suction pump immediately following immersion of the buds in the fixing solution.

Several fixing agents were used, among them various modifications of Navashin's solution, Flemming's medium and stronger solutions, chromo-acetic-osmic solution, Carnoy's solution, and corrosive sublimate. The modifications of Navashin's solution gave the most satisfactory results.

Sections were cut from 4 to 12  $\mu$ , those about 6  $\mu$  proving most satisfactory for the study of both nuclear and cytoplasmic division. The preparations used for the study of meiosis were stained in iron-alum haematoxylin, while those used in the study of cytokinesis were counterstained with orange G. Owing to the strong affinity of the mother cell wall for the crystal violet, Flemming's triple stain proved unsatisfactory for preparations designed to show cell division.

### Observations

#### RESTING NUCLEUS TO DIAKINESIS

In the resting condition the pollen mother cells are polyhedral in shape. Usually two pollen mother cells, separated by a single layer of sterile cells, occur across the diameter of the locule. The nuclei now have a diameter of approximately 8  $\mu$ . The strands of the reticulum are thin, and where they cross and anastomose, the chromatin is aggregated in thick masses. There is usually one large deeply staining nucleolus, rarely two of unequal size.

As the prophases begin, the nuclei of the mother cells enlarge rapidly, the chromatin network becomes more threadlike, the aggregations spread out along the threads, and a smoother and more uniform appearance is gradually assumed. Soon after these changes occur the threads pair side by side.

The synizetic condition now ensues; but, contrary to the usual condition, in which the synizetic knot lies on the side of the nucleus closest to the mother cell wall, in *Asimina triloba* the knot is on the

side of the nucleus farthest from the wall. The nucleolus is commonly not in contact with the synizetic knot, but lies free within the nuclear cavity. It is always spherical at this time, and may show small peripherally arranged vacuoles. During the changes just described the nuclei have enlarged gradually and continue to do so until at pachytene they have a diameter of approximately  $16\ \mu$ .

Following synizesis, the chromatin threads, consisting of double strands closely twisted about each other, begin to spread throughout the nuclear cavity. In some cases this association was so close that their double nature could not be detected, but at other places the twisting of the strands was clearly evident.

As the pachytene condition begins, the threadlike mass continues to loosen and spreads throughout the entire nuclear cavity. During early pachytene there is little evidence of the double nature of the strands; but as they pass into diplotene, the paired elements become less closely associated. The threads continue to shorten and assume the typical form and scattered arrangement of bivalent chromosomes which characterize diakinesis. Counts made of bivalent chromosomes at diakinesis indicated a haploid number of nine, although it is not possible to make a definite statement concerning their number. During the anaphases of the second meiotic division nine chromosomes were observed passing to the poles of the two daughter cells, thus tending to confirm this number as being the correct count.

#### CYTOKINESIS

FIRST DIVISION.—Following diakinesis, the mother cell wall continues to thicken uniformly until at the time the microspores are formed it has a thickness of approximately  $6\ \mu$ . The spherical pollen mother cells lie free in the anther locule. During diakinesis, the nuclear membrane disappears and the fibers of the diarch spindle, which appears at about this time, become attached to the chromosomes. As the chromosomes pass toward the poles, the mother cell becomes elongated in the direction of the axis of the first division. Occasionally the mother cells begin elongation before the end of the prophase. During interkinesis, the chromatin material of the daughter nuclei does not become reorganized into a definite reticu-

lum, but assumes the form of spherical, irregular, or threadlike masses (figs. 1-5, 7-9). The daughter nuclei are discoid and usually somewhat flattened on the side toward the region of the first cytoplasmic constriction (figs. 3-5, 7-9). After the daughter nuclei have been formed, the spindle spreads out until it extends almost across the mid-plane of the cell (fig. 2). The spindle fibers are thicker and more irregularly arranged than during the anaphases; and as cytokinesis occurs, they gradually become more granular.

The first evidence of cytokinesis is the appearance of a constriction in the equatorial region and in a plane approximately equidistant from the daughter nuclei (fig. 2). The furrow is usually initiated after the daughter nuclei have been formed; in some cells, however, the furrow is initiated during the anaphases of the first meiotic division. When the furrow first begins to form it is narrow and pointed, but soon thickens considerably (fig. 3). The advancing edge of the furrow becomes broad and round, giving the cell a two-lobed appearance, and from the time it is initiated it is completely filled with material in close apposition to the mother cell wall.

In no cell observed was there any indication of a cell plate prior to the initiation of the furrow; but when a cytoplasmic isthmus of approximately one-third the diameter of the mother cell remains between the advancing edges of the furrow, an inconspicuous cell plate may be formed in the mid-region of the spindle (figs. 3-5). This condition occurred in a number of the cells observed in this stage of development; in others there was no indication of such a structure.

The furrow continues to deepen; and when the isthmus remaining is about one-fifth the diameter of the mother cell in the direction of the constriction, the cell plate, if formed, has completely disappeared (figs. 6-8). Thus the cell plate formed here is an evanescent structure and plays no part in the furrowing process. The spindle fibers have now disappeared also, and nothing was observed to indicate that they play a part in the formation of the developing plasma membranes, as suggested by FARR (2).

Within a short time the cytoplasmic isthmus is completely severed by the constriction of the plasma membrane, and the wall of the

mother cell forms a continuous structure between the two daughter cells (fig. 9). The wall which develops between the daughter cells is about three times as thick as the mother cell wall surrounding the two cells and, as shown by microchemical tests, consists of callose.

Since there is no definite correspondence between nuclear phases and stages in the furrowing process, it is not possible to arrange a series of stages in cytokinesis on the basis of nuclear changes. In the majority of cells the first cytoplasmic division is completed before the second nuclear divisions occur, although occasionally the latter may occur during the process of the first constriction.

SECOND DIVISION.—The spindles of the second nuclear division figures now form and are usually parallel with one another and perpendicular to the axis of the spindle of the first division. Figure 10 shows a cell during the anaphases of the second nuclear division with nine chromosomes passing to each pole. Following this the spindles disappear, and no suggestion of a cell plate was observed in any of these division figures.

The furrow of the second cytoplasmic division begins along the inner periphery of the daughter cells adjacent to the wall of the first division (fig. 11). It proceeds to about one-third the diameter of the cells and now begins to appear along the outer peripheral wall (fig. 12). Furrowing then proceeds on all sides at about the same rate, and constriction is completed at a point closer to the outer peripheral wall than to the wall which comes in following the first constriction furrow (fig. 13). In figure 14 a condition is shown in which one of the daughter cells is dividing more rapidly from the outer wall, and constriction of one of the daughter cells will be completed closer to the wall of the first division than to the mother cell wall. Usually the second divisions are completed at approximately the same time, but occasionally one of the daughter cells may divide before the other. One of the daughter cells may undergo the second cytoplasmic division before second nuclear division has been initiated in the sister cell (fig. 15).

The two successive divisions of the pollen mother cell form four microspores which are quadrilaterally arranged (fig. 16). The locules of the anther are of such small diameter that one quartet of

microspores fills the entire space. After the second division is complete, the nuclei lie close to the plasma membrane of the microspores, but not in contact with it. After a time they become centrally placed.

#### DEVELOPMENT OF POLLEN GRAIN

The young microspores, with dense granular cytoplasm, measure approximately  $30\ \mu$  in diameter (fig. 16). They soon begin to enlarge, apparently with little increase, if any, in the amount of cytoplasm at this time. With the increase in size the vacuoles enlarge and the cytoplasm remains for a time distributed throughout the cell. At the time the microspore has reached its full size, the cytoplasm lies mainly at one side, having one or a few large vacuoles in the central portion of the cell (fig. 17). During enlargement of the microspore its wall increases in thickness, developing a pectose intine layer and an exine layer of undetermined composition. After a time the cytoplasm begins to increase in volume; simultaneously the vacuoles become smaller and more numerous, until the cytoplasm of the microspores finally appears densely alveolar (figs. 18-21).

The nucleus of the microspore now divides, thereby forming the generative and the tube nuclei of the young microgametophyte (fig. 21). This division may occur before the cytoplasm has become densely alveolar (fig. 20). The generative nucleus is smaller than the tube nucleus, and it, with a small amount of cytoplasm, is set off from the tube cell by a membrane. When the binucleate condition of the microgametophyte occurs the tapetal tissue has disappeared and the anthers have opened for the shedding of pollen.

#### Discussion

FARR (2) gives an extensive review of the literature dealing with cytokinesis in pollen mother cells. His results tend to indicate that the divisions of the pollen mother cells, in most angiosperms, are brought about by furrowing rather than by cell plates. In general, bipartition is common to the monocotyledons and quadripartition to the dicotyledons, but intermediate types of microspore formation have been reported for a few scattered forms. Some of those which are similar to *Asimina* will be discussed briefly.

ROSANOFF, in his work on *Acacia paradoxa*, gives two figures (6, figs. 24, 25) showing bipartition of the pollen mother cells by furrowing. After completion of the first division, the furrowing appears along the periphery of the daughter cells and later along the wall of the first division furrow. Cell plates are not shown, and he does not discuss the process of cytokinesis. From these two figures it appears that the division in *A. paradoxa* is similar to that which occurs in *Asimina triloba*. It differs, however, in that in the former the furrow of the first division appears first along the outer periphery of the daughter cells, while in *Asimina* it appears first along the inner periphery of the daughter cells adjacent to the wall of the first division furrow.

In his study of *Magnolia*, FARR (3) reported that the first cytoplasmic division of the pollen mother cells begins during interkinesis and continues until the isthmus of cytoplasm which remains is about one-half the diameter of the mother cell. Division is now arrested, the fibers disappear, and the second nuclear divisions occur. During interkinesis, the furrow of the first division may almost disappear. The furrow of the second division appears at the periphery of the cell and at right angles to the axis of the spindle of the first division. The spindle of the first nuclear division has now reappeared. The furrow of the second division deepens more rapidly than the furrow of the first division so that both constrictions are completed at approximately the same time. There was no indication of a true cell plate after the initiation of the constriction. FARR suggested that furrowing might have been brought about by attraction between the nuclear and cytoplasmic membranes.

FARR (2) observed that the spindle fibers come in contact with the plasma membrane at the advancing edge of the furrow, and suggested the possibility of their being used in the formation of the developing plasma membranes. The spindle fibers in *Asimina* were not observed to come in contact with the plasma membrane as the furrow advanced.

SAMUELSSON (7) reported a type of cytokinesis in *Anona cherimolia* that is similar to that in *Asimina triloba*. Division is by successive constriction, and the mother cell is divided into two daughter cells. The furrow begins during the telophases of the first nuclear



division and is completed after the second nuclear divisions have occurred. During the metaphases of the second nuclear division, the cells are connected by a narrow bridge of cytoplasm. The second division takes place in the same manner as the first. *Asimina* differs from *Anona* in that there is not so close a correlation between nuclear phases and stages in the furrowing process in the former as occurs in the latter. The second division in *Asimina* differs from the second division in *Anona* in that (as already shown) it does not agree completely with the first division as SAMUELSSON described for *Anona*. SAMUELSSON thought that the type of division that occurred in *Anona* was more closely related to the monocotyledonous than to the dicotyledonous type.

SAMUELSSON reported a similar type of division in *Aristolochia clematitis*. Immediately after the first cytoplasmic division the intervening wall thickens greatly. This was observed to be true in *Asimina*. In *Aristolochia* the spindles of the second division may not always be parallel to one another and perpendicular to the axis of the spindle of the first division. The four microspores may be arranged in a straight line as a result of the spindles of the second division being in the same plane as the spindle of the first division. In some cells he reported one of the spindles of the second division to be parallel with the spindle of the first division and the other perpendicular to it, the two spindles of the second division being in the same plane.

HERMS observed the pollen mother cells in *Asimina triloba* to be differentiated in early October, and stated that they remain in this condition until the middle of January. He reported that on March 10 the pollen mother cells begin to enlarge, and by April 14 a bridge of tissue is formed between the microsporocytes. Spore tetrads are formed by April 21. By May 5 pollen is ready to be shed. Similar definite dates were given for the stages in development of the endosperm and megagametophyte. My own observations indicate that it is not possible to associate stages of development with precise dates because of the great variation in the different buds used for study. Floral buds were collected in late March in which pollen mother cells had not become differentiated. Also, in some collected as late as May 1, microspores had not been formed.

### Summary

1. During meiosis the chromatin material of the pollen mother cells undergoes parasynaptic association.
2. At synizesis the chromatic knot lies on the side of the nucleus farthest from the mother cell wall.
3. The haploid number of chromosomes is apparently nine.
4. Cytokinesis of the pollen mother cells is by successive constriction. Evanescent cell plates may occur in the first division.
5. In general the first cytoplasmic division is completed before the second nuclear divisions occur.
6. In the second cytoplasmic division the furrow appears first on the side of the daughter cells adjacent to the wall of the first division, and proceeds outward for a time before it appears at the outer periphery of the cell.
7. The young microspores are relatively small and have a quadrilateral arrangement as a result of the spindles of the second division being perpendicular to the axis of the first division and in the same plane with it.
8. Before being shed, the microspores develop into relatively large binucleate microgametophytes which are completely filled with dense, alveola-like cytoplasm.

The writer expresses appreciation to Dr. J. M. BEAL for suggestions and constructive criticism throughout the course of this investigation.

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#### EXPLANATION OF PLATE I

FIG. 1.—Pollen mother cells showing first nuclear division.

FIGS. 2-9.—Pollen mother cells showing successive stages in first cytoplasmic division.

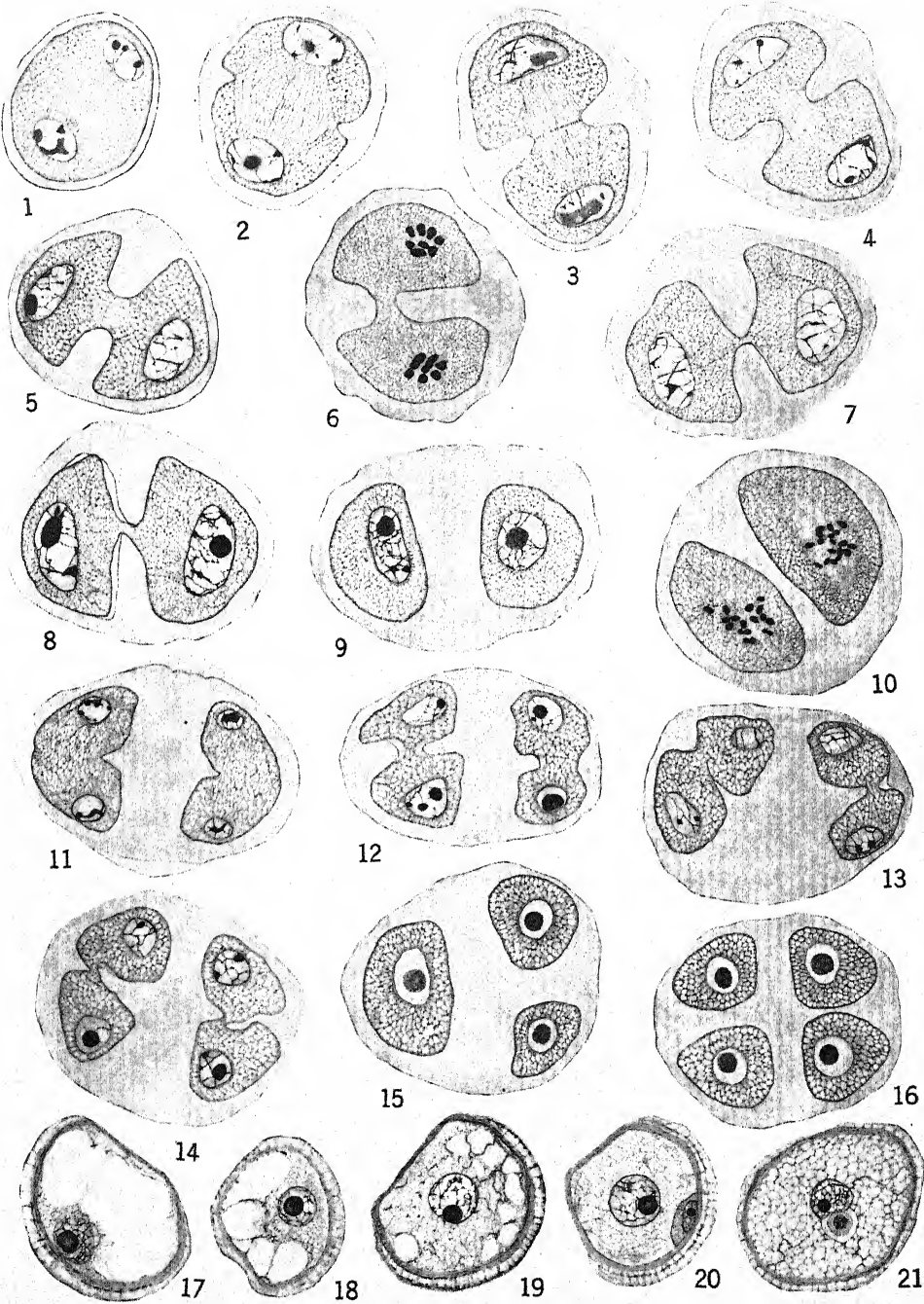
FIG. 10.—Daughter cells in early anaphases of second nuclear divisions with nine chromosomes passing to each pole.

FIGS. 11-14.—Daughter cells showing successive stages in second cytoplasmic division. Fig. 14, unusual condition in one daughter cell in which the furrow has proceeded more rapidly from outer periphery toward wall of first division.

FIG. 15.—One daughter cell which has undergone second cytoplasmic division before the second nuclear division of sister cell has been initiated.

FIG. 16.—Quartet of microspores quadrilaterally arranged.

FIGS. 17-21.—Successive stages in development of pollen grain from mature microspore.



LOCKE on ASIMINA



## DEVELOPMENT OF THE MALE GAMETES OF LILIUM<sup>1</sup>

D. C. COOPER

(WITH THIRTY-ONE FIGURES)

### Introduction

Differing observations have been made regarding the history of the male gametes in the Liliaceae, and especially in the genus *Lilium*. The present investigation was undertaken because the findings of certain recent investigators are almost diametrically opposed to those of earlier workers. An attempt has been made to get a series of stages, from generative cell to fertilization, in the development of the male gametes of *Lilium regale* Wilson. For comparative purposes, material of *L. auratum* Lindl. and *L. philippinense* Baker was also studied.

**MATERIALS AND METHODS.**—In order to obtain stages of the division within the generative cell, stigmas of *Lilium regale* and of *L. auratum* were pollinated and the material (stigma and style) collected at intervals between 48 and 72 hours after pollination. Some of these stigmas and styles were split lengthwise, fixed in Karpechenko's modification of Navashin's fluid, imbedded in paraffin, sectioned, and stained to show the pollen tubes in the stylar canal. In other cases the pollen tubes were gently scooped from the stylar canal on to a clean slide and fixed for one hour in a modification of Carnoy's solution (95 per cent alcohol, 6 parts; glacial acetic acid, 2 parts; chloroform, 2 parts). These were then washed in 95 per cent alcohol and mounted in aceto-carmine.

Slides bearing pollen tubes were also placed in Karpechenko's fixing fluid, washed and stained by the iodine-crystal violet-picric acid method. When the extremely delicate pollen tubes were removed from the stylar canal, care was taken not to pull or stretch them.

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Another method of obtaining pollen tubes was to germinate the pollen grains on a slide. The sticky exudate of the stigma was smeared on a clean slide and pollen dusted thereon. Such slides were placed in a moist chamber for 36 to 48 hours, then fixed in Karpechenko's fluid and stained either in Delafield's haematoxylin or by the iodine-crystal violet process. Pollen of *Lilium regale*, *L. auratum*, and *L. philippinense* was thus germinated and treated.

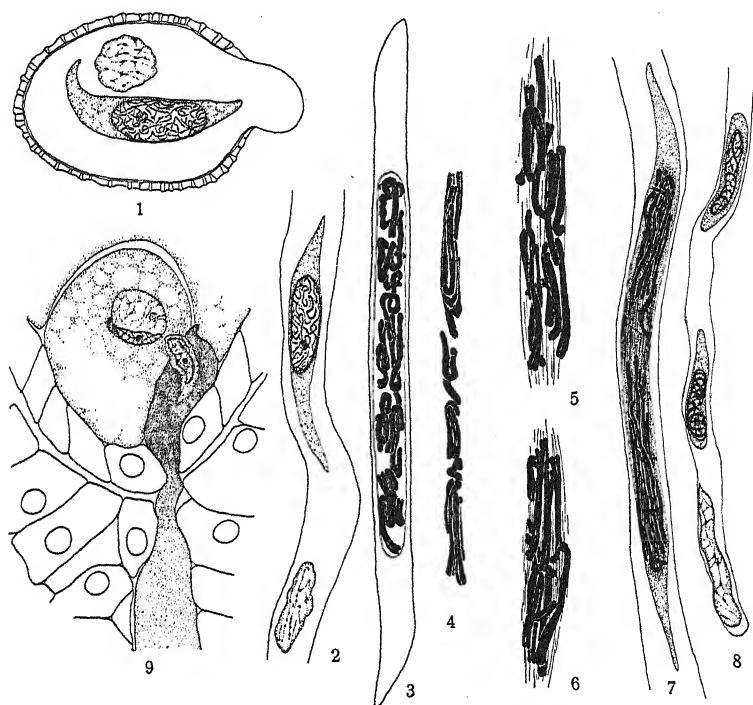
### Observations

*Lilium regale*.—The pollen tube appears at a fissure of the ruptured exine (fig. 1) and grows downward between the papillate hairs of the stigma until it reaches the epidermis. It then bends toward and enters the stylar canal and grows along the canal toward the ovary.

The nucleus of the tube cell usually precedes the generative cell into the pollen tube (fig. 2), although it may lie beside or follow the generative cell. The tube nucleus is irregular in shape and structure (figs. 2, 8). When it lies beside the generative cell it is usually drawn out into a dumb-bell shape in consequence of the small diameter of the pollen tube.

At the time of germination of the pollen grain, the nucleus of the generative cell is in an early prophase stage (fig. 1). This cell and its nucleus become markedly elongate upon entering the pollen tube (fig. 2). During the period in which the pollen tube is growing along the stigmatic surface and down the funnel-like upper end of the stylar canal, the deeply staining chromosomes are formed, each of which shows a longitudinal split (figs. 3, 10). Shortly after the pollen tube and the included generative cell have passed into the narrow neck of the stylar canal (from 48 to 60 hours after pollination), the nuclear membrane disappears (figs. 5, 6, 12-15). A typical spindle is present. The figures are elongated because of the length of the chromosomes and the small diameter of the pollen tube. The longitudinally split chromosomes divide on the equatorial plate (figs. 16, 17). Figures 7 and 18 show late anaphase stages with twelve chromosomes at each pole. A cell plate is formed across the mid-region of the spindle, and two separate gametes (cells) are formed. The nuclear material of each daughter nucleus spreads out into a

chromatic network approximating that of a so-called resting stage (figs. 8, 9, 19). The zone of cytoplasm about each male gamete nucleus persists until fertilization. Figure 9 shows a stage in fertilization, the male gamete nucleus near the egg nucleus having now escaped from its cytoplasmic sheath, since no such sheath can be



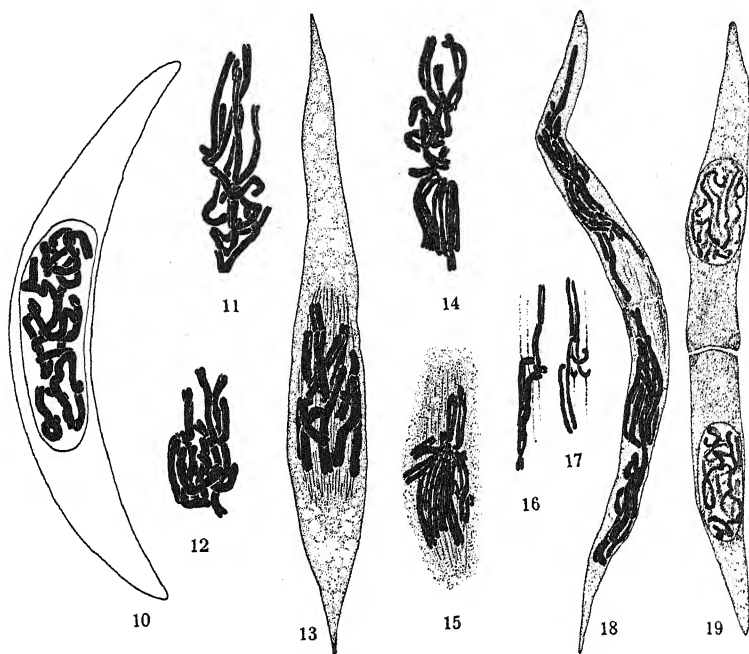
FIGS. 1-9.—*Lilium regale*, stages in division of generative cell as found in pollen tubes growing in stylar canal: Fig. 1, germinating pollen grain;  $\times 400$ . Fig. 2, generative cell and tube nucleus in pollen tube;  $\times 400$ . Fig. 3, generative cell; nucleus in late prophase stage of division;  $\times 780$ . Fig. 4, chromosome advancing to equatorial plate;  $\times 780$ . Fig. 5, early metaphase;  $\times 780$ . Fig. 6, metaphase;  $\times 780$ . Fig. 7, late anaphase showing early stage of cell plate formation;  $\times 780$ . Fig. 8, tip of pollen tube containing tube nucleus and two male gametes;  $\times 400$ . Fig. 9, fertilization; one male gamete nucleus near egg nucleus, second leaving ruptured pollen tube;  $\times 780$ .

discerned. The second male gamete (still a complete cell with nucleus and cytoplasm) is just leaving the ruptured pollen tube.

When the pollen tubes are grown in culture, the generative cell



(figs. 10-17) divides in a manner very similar to that just described. The cell and nucleus are not so elongated, however, and the diameter of the pollen tube is somewhat greater than when it grows in the stylar canal. The chromosomes are shorter and thicker in culture, but under either condition of growth they have similar



FIGS. 10-19.—*Lilium regale*, stages in division of generative cell as found in pollen tubes grown in culture: Fig. 10, generative cell; nucleus at late prophase stage;  $\times 780$ . Figs. 11, 12, chromosomes advancing to equatorial plate;  $\times 780$ . Fig. 13, early metaphase;  $\times 780$ . Figs. 14, 15, metaphase;  $\times 780$ . Fig. 16, three chromosomes at metaphase;  $\times 780$ . Fig. 17, two chromosomes at early anaphase;  $\times 780$ . Fig. 18, late anaphase showing cell plate formation;  $\times 780$ . Fig. 19, two male gametes; cell plate completed;  $\times 780$ .

morphological features. As seen in the equatorial plate, they vary in length so that the longest chromosome is approximately 2.5 times as long as the shortest. Three of the chromosomes have median or submedian spindle fiber attachments; nine have subterminal attachments (fig. 5). In these respects the appearances are similar to those

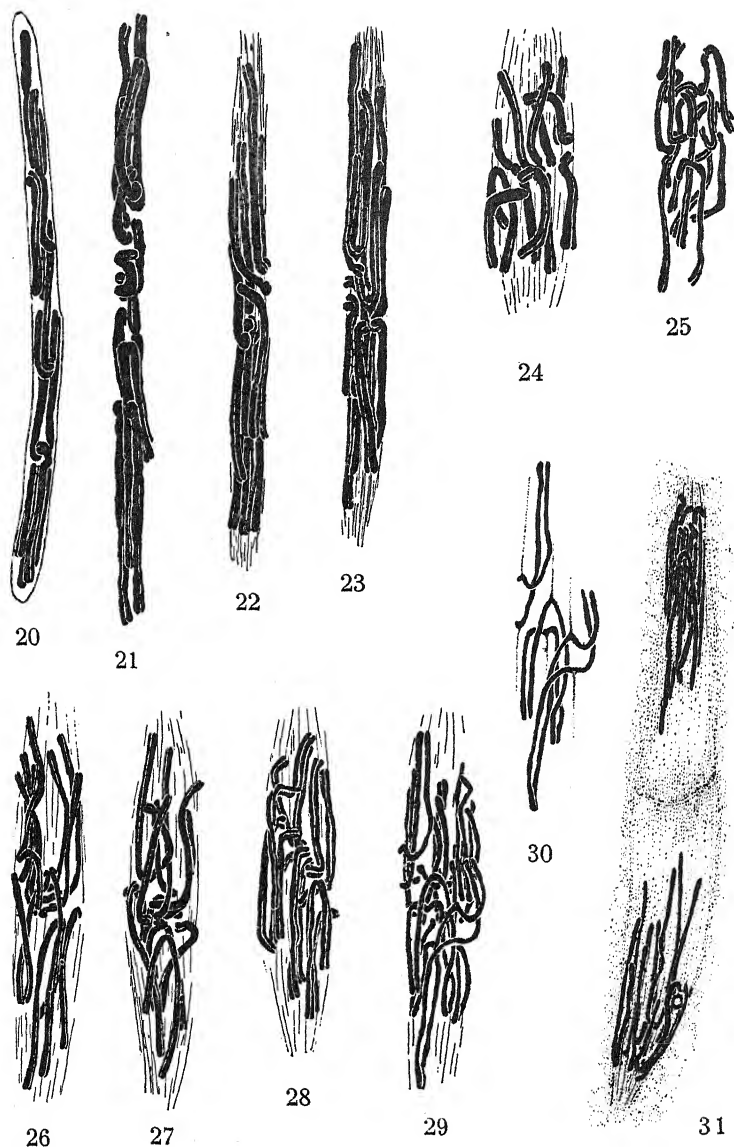
seen in the haploid divisions in the formation of the embryo sac of *L. henryi* (1). A somewhat shorter spindle occurs in the material grown in culture (figs. 13-15) as compared with material found in the stylar canal. Cell plate formation (fig. 18) likewise occurs and two male gamete cells are formed (fig. 19).

*Lilium auratum*.—The division of the generative nucleus in this species resembles that described for *L. regale*. The chromosomes of the elongated nucleus (fig. 20) as found in pollen tubes located in the stylar canal are drawn into an equatorial plate (figs. 21, 22), and the division figure is typical, a well formed spindle being present (figs. 22-23). A late prophase (fig. 20) shows the longitudinally split chromosomes, which retain their double nature until the halves separate in the equatorial plate. In those pollen tubes grown on the stigmatic exudate in a moist chamber, the chromosomes are shorter and thicker and the spindle is more definite (fig. 24).

*Lilium philippinense*.—The material of this species examined was from pollen tubes grown in culture. Figure 25 shows the chromosome complement of the generative nucleus shortly after the dissolution of the surrounding membrane. These chromosomes pass on to a typical mitotic spindle (figs. 26-28). At the equatorial plate stage (fig. 28) the spindle fiber attachment regions are approximately in the equatorial plane of the spindle. Each chromosome begins to divide at the spindle fiber attachment region (fig. 29). This is clearly evident in figure 30, showing three of the chromosomes of the complex in the preceding figure. Two of these chromosomes have approximately median spindle fiber attachments; one has a subterminal attachment. In the anaphase (fig. 31) the chromosomes are elongated and the median and subterminal spindle fiber attachments are clearly evident. In the cell represented in this figure a cell plate is being formed.

### Discussion

The method of division of the generative nucleus and generative cell of the three species of *Lilium* herein described is in agreement with the observations of a number of investigators of other members of the Liliaceae [*Lilium martagon* (2, 4, 8); *L. candidum* (3); *Hemerocallis flava* (9)]. These observations are at variance with those of



FIGS. 20-31.—Stages in division of generative cell taken from pollen tubes growing in stylar canal. *Lilium auratum*: Fig. 20, late prophase; fig. 21, chromosomes advancing to equatorial plate; fig. 22, early metaphase; fig. 23, late metaphase; figs. 24-31, stages in same division as found in pollen tubes growing in culture; fig. 24, metaphase. *L. philippinense*: Fig. 25, chromosomes advancing to equatorial plate; figs. 26, 27, early metaphases; fig. 28, late metaphase; fig. 29, early anaphase; fig. 30, three of chromosomes of fig. 28 showing spindle fiber attachments; fig. 31, late anaphase showing cell plate formation. All  $\times 1025$ .

certain other workers who found no signs of a spindle in this division. NAVASHIN (5) observed no stage in *L. martagon* which might be interpreted as a typical metaphase. In the earlier stages of division, however, he observed longitudinally split chromosomes. His figures 7 and 14 are interpreted as equatorial plate stages. They are similar to the late prophases of *L. regale* and *L. auratum* shown in figures 4 and 21 respectively, where the chromosomes are advancing to the equatorial plate. Since NAVASHIN saw no spindle fibers, he concluded that the chromosomes pass to the telophase position by some type of movement peculiar to themselves.

WELSFORD (10) also saw no spindle at any stage of this division in either *Lilium auratum* or *L. martagon*. She found the division process similar in these two species and described it for *L. martagon* in detail. Her figure 16, which approximates an equatorial plate stage, and figure 17, also entitled an equatorial plate stage, are likewise similar to my figures 4 and 21. TRANKOWSKI (9) observed a metaphase without a spindle in *Convallaria majalis*.

O'MARA (6) examined the corresponding division in *Lilium regale* and concluded that a typical equatorial plate is lacking since he could not demonstrate the presence of spindle fibers. An examination of his figures shows that most of his observations are based on configurations similar to my figures 4, 5, and 11, which must be interpreted as late prophases rather than as equatorial plate stages (figs. 6, 13-15, 22-24, 26-29). That these are late prophase configurations is all the more probable when it is considered that his material was collected between 24 and 36 hours after pollination, whereas the material showing equatorial plate stages in the present study was collected 48 to 72 hours after pollination. In the case of pollen germinated in culture, the stages shown in figures 10, 11, and 12 were obtained from material fixed after 36 hours in the moist chamber, while abundant configurations showing equatorial plates, anaphases, and telophases were present in cultures which were kept in a moist atmosphere from 42 to 48 hours. O'MARA's figures 2, 4, 12, and 13, termed metaphases, are similar to my figures of stages at which the chromosomes are passing to the equatorial plate. His figure 8 approximates the late prophase stage shown in figure 5 of *L. regale* and in figure 22 of *L. auratum*. In the three species of

*Lilium* here discussed, the division of the generative nucleus appears to be regular.

Differing observations have been made as to the nature of the male gametes of the Liliaceae. GUIGNARD (2) observed the male gametes to be distinct cells in *L. martagon*, whereas both KOERNICKE (4) and STRASBURGER (8) found only male gamete nuclei in that species. STRASBURGER described the formation of an evanescent cell plate which disintegrates, leaving naked gamete nuclei in the cytoplasm of the pollen tube. WELSFORD (10) in *L. auratum* and *L. martagon*, and HERRIG (3) in *L. candidum*, showed that the male gametes exist as distinct cells in these species. WELSFORD found that the male gametes lose their cytoplasm upon entering the embryo sac. TRANKOWSKI (9) reported division of the generative cell to be by constriction in *Convallaria majalis*; the cytoplasm of each male gamete, according to him, soon disappears, leaving naked nuclei. O'MARA (6) found some evidence of constriction in this division in *L. regale*. According to him, the gamete cytoplasm dissolves and at later stages two naked nuclei are present in the cytoplasm of the pollen tube. The division of the generative cell is by means of cell plate formation in *Lilium regale* and *L. philippinense*. In all three species studied the male gametes exist as distinct cells. In the case of *L. regale*, the sheath of cytoplasm around each male gamete nucleus was observed to persist until fertilization.

Varying observations have been made in other families of plants regarding the nature of the division of the generative cell and the type of male gamete present (9). Mitotic figures have been described in the generative cell and many cases are known in which the male gametes exist as distinct cells.

### Summary

1. The nucleus of the generative cell in *Lilium regale*, *L. auratum*, and *L. philippinense* passes through a typical mitotic division in the production of the two male gametes.
2. A distinct spindle is present.
3. Three of the chromosomes have median or submedian spindle fiber attachments; the other nine chromosomes have subterminal attachments.

4. The generative cell is divided by means of a cell plate.
5. The male gametes are fully organized cells.

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## ANATOMICAL STUDY OF THE SEEDLING OF HIBISCUS TRIONUM

ANNA MAY KIMMELL

(WITH TWENTY-FIVE FIGURES)

### Introduction

A study of *Hibiscus trionum* L., which is a common weed in the state of Nebraska and elsewhere, was made to determine the details of root-stem transition. Some work has been done on related species. GERARD (3) and THOMAS (8), working with *Althaea rosea*, described the normal seedling as typical of the "Anemarrhena" group; but they also found that the seedling might exhibit considerable variation in its method of transition. In 1926 BEXON (1) continued this work.

**MATERIALS AND METHODS.**—The plants were grown from seeds collected in the fall and winter at Ames and Lincoln, Nebraska.

Formalin-acetic-alcohol was used as fixing agent for the various stages of development. Each seedling was cut into segments approximately 7 mm. long in order that the fixative might thoroughly penetrate the tissues. The material remained in the fixative at least 10 days before dehydration. Safranin and gentian violet were used as stains. Freehand sections of a great number of seedlings were cut to determine the typical symmetry. These were dehydrated in alcohol and mounted in sandarac. Whole seedlings were cleared in cedar oil.

**GERMINATION.**—To overcome the difficulty in germinating seeds in the usual way, several methods were tried. Cutting the seed coat with a sharp razor blade resulted in abnormal seedlings. Freezing and burning were found to be unsatisfactory, but treating the seeds with concentrated sulphuric acid for 20 minutes resulted in seedlings which were perfect in all respects (fig. 1). Seeds left in acid for 25 and 30 minutes had 100 per cent germination but did not develop normally. In 48 hours, 16 per cent exhibited a maximum length of 14 mm. The average length was 5 mm. and the minimum was 0.5

mm. These seedlings were undoubtedly injured by the penetration of the acid into the embryos. The radicles appeared stunted and there was no development of root hairs.

### Investigation

Seedlings of three different ages were used: 5, 16, and 24 days old. The root-stem transition could be interpreted best in the 5-day seed-

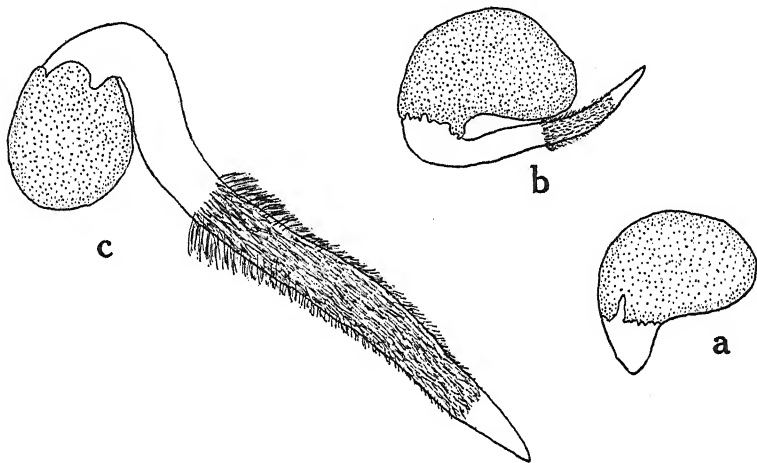


FIG. 1.—Germinating seeds in various stages of development: *a*, radicle emerging from seed coat; *b*, root 5 mm. long with root hairs; *c*, seed with root 13 mm. long, root hairs abundant.

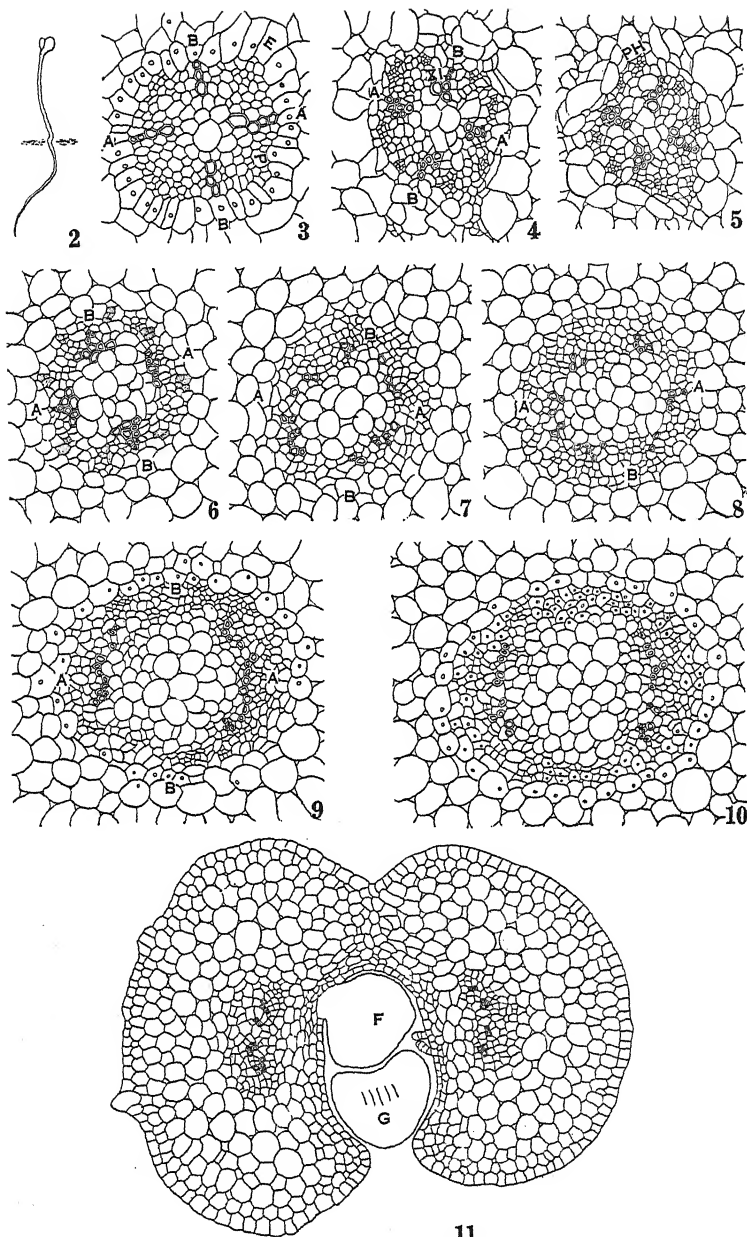
lings. Older seedlings were used to study the stem structure and the vascular system of the first leaves.

From stem tip to root tip a 5-day seedling measures approximately 50 mm. (fig. 2). The two cotyledons are then completely unfolded, one of the laminae being slightly larger than the other, but the first foliage leaf is not visible. The hypocotyl is covered with many stellate and glandular hairs.

The root is tetrarch (fig. 3). The pericycle (*P*) is intersected at four points where the protoxylem abuts the endodermis. The cells of the endodermis vary from the radially elongated ones (*E*, fig. 3) to irregular cells that are difficult to follow in transverse section (figs. 4, 5). The cortex is from seven to ten cell layers in thickness.

Sections at slightly higher levels (figs. 4, 5) show that two phloem





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FIGS. 2-11.—Figs. 2-10, drawings of 5-day-old plant and from serial sections through root and hypocotyl (*A-A*, cotyledonary plane; *B-B*, intercotyledonary plane): fig. 2, 5-day-old seedling ( $\times \frac{1}{2}$ ); fig. 3, tetrarch stage; fig. 4, eight phloem groups; fig. 5, endodermis irregular; fig. 6, differentiation of metaxylem forming xylem arcs; figs. 7, 8, differentiation of each arc into two or more distinct groups; figs. 9, 10, intercotyledonary plane conspicuous *B-B*.  $\times 100$ . Fig. 11, sections cut slightly above cotyledonary node of 5-day-old plant (*F*, bud with meristematic cells; *G*, petiole of first foliage leaf).  $\times 100$ .

groups (*PH*) have developed in each angle formed by the xylem rays.

The "xylem intermediary" (*XI*) as described by CHAUVEAUD (2) is present at this level (figs. 4, 5). At a higher level (fig. 6) the metaxylem elements are differentiated in the shape of arcs. Some of the protoxylem vessels in the radial position are distorted and partially disintegrated.

During the subsequent processes of growth, some of the protoxylem elements become obliterated and the compact arcs of the metaxylem (fig. 6) change in appearance, each arc becoming separated into two more or less distinct groups (figs. 7, 8). At this level the intercotyledonary plane cannot be determined. Soon the vascular elements form an interrupted oval (fig. 9) and growth in the intercotyledonary plane (*B-B*) divides the elements of the two intercotyledonary polar groups. Half of the vascular tissue of each goes to each cotyledonary plane, and there occupies a lateral position with respect to the polar protoxylem elements of that plane. The xylem elements at this level are largely protoxylem, consisting of vessels with annular and spiral thickenings. There are only a few tracheids.

Because of the reorientation of vascular tissues in the transition region, the xylem in the cotyledonary plane, near the point of divergence of the cotyledonary petioles, is made up of the polar or main xylem group of that plane and a part of each of the polar xylem groups of the intercotyledonary plane. Owing to the disappearance of some polar protoxylem elements and the addition of the portions of the two intercotyledonary polar xylem groups, the cotyledonary trace consists of four distinct groups of xylem (fig. 10). In the cotyledonary trace each of these xylem groups lies internal to the phloem group so that the bundles are collateral. As such a cotyledon is separated from the cotyledonary node, a line of cleavage in the parenchyma is formed. The cotyledons show the alternate phyllotaxy which is characteristic of this plant. In a given transverse section the vessels of one cotyledon are seen in transverse section while the vessels of the other appear in longitudinal section. This is also evident externally at the cotyledonary node (fig. 11), as one petiole is larger than the other.

The entire vascular system of the cotyledons is continuous with the primary vascular system of the root. The cotyledonary trace consists of the "double bundle" and two laterals. The "double bundle," first described by THOMAS (7), is comparable with her second type which is sometimes represented by two distinct bundles which together constitute one pole of the root. In addition to the two central bundles, there is frequently present a lateral bundle on each side, so that there may be four bundles in each cotyledonary trace. The condition found here is similar to that described by THOMAS (7) for *Althaea*.

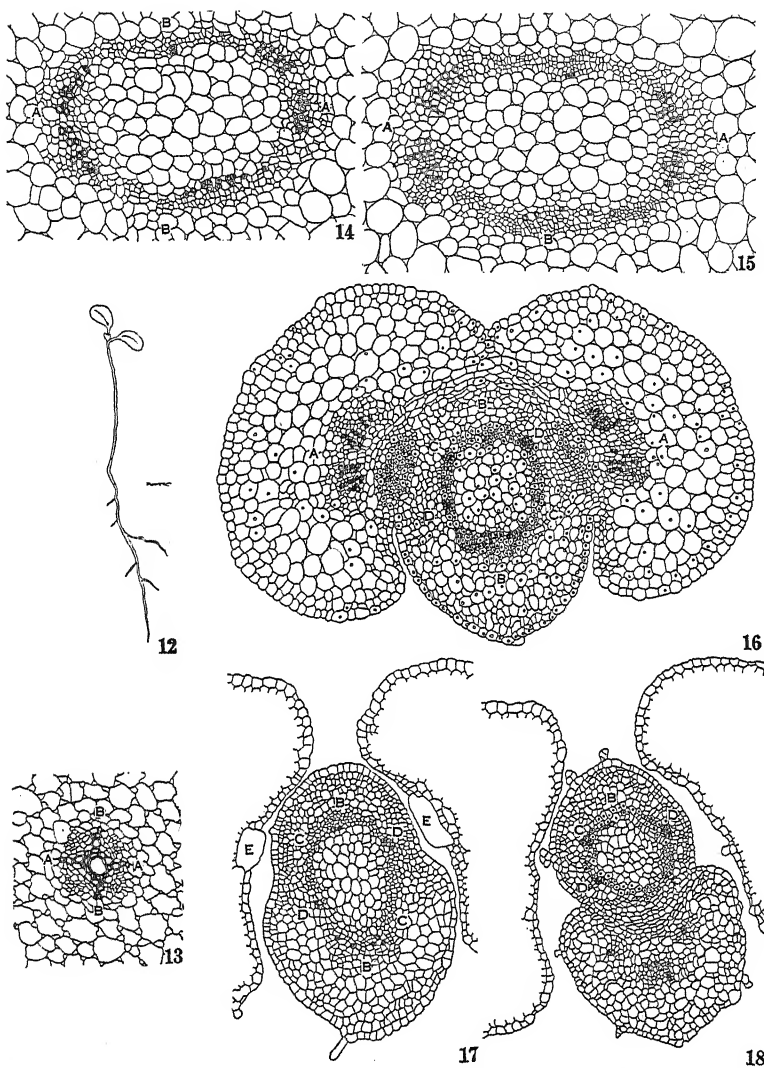
The bud apex of the epicotyl (*F*) lies between the petioles of the two cotyledons. On the intercotyledonary side of this stem meristem the first foliage leaf is differentiated as a protuberance of tissue. In this rounded mass of tissue are differentiated rows of radial procambial strands which form the trace of the first leaf. As the petiole of this leaf is diverged from the epicotyl, the undifferentiated meristem (*G*) may be seen in the center, arranged in linear rows (fig. 11).

#### DIFFERENTIATION OF FIRST AND SECOND LEAVES

Seedlings 16 days old measured approximately 100 mm. from root tip to stem tip.

In addition to the primary xylem elements of the cotyledonary plane, vessels which have developed from the intercotyledonary meristem, so prominent in figure 10, are evident (figs. 14, 15). On one side are a number of vessels, while on the other there are only two (fig. 14). The meristematic ring of the hypocotyl becomes more and more elliptical in shape, owing to extensive growth in the intercotyledonary plane (fig. 15). As the first cotyledonary petiole is cut off, the meristematic cells of both intercotyledonary planes are united on that side. The parenchyma cells in the cotyledonary gap are meristematic and form a bud (*E*) in the axis of the cotyledonary petiole (figs. 16, 17).

At this stage the meristem in the region of the epicotyl is horse-shoe-shaped, but is again cylindrical above the point of divergence of the second cotyledonary petiole from the main stem (fig. 16). A bud is formed in this cotyledonary gap (figs. 16, 17). At a higher



FIGS. 12-18.—Figs. 12-15, 16-day-old plant ( $\times \frac{1}{2}$ ) and drawings from series of transverse sections: fig. 13, protoxylem and metaxylem in tetrach root; figs. 14, 15, vessels of epicotyl in intercotyledonary plane *B-B*.  $\times 100$ . Fig. 16, 16-day-old plant, transverse section at cotyledonary node showing petioles of cotyledons and epicotyl with its continuous meristematic ring.  $\times 70$ . Figs. 17, 18, transverse sections through epicotyl (*C-C*, diagonal plane; *D-D*, diagonal plane; *E-E*, meristematic buds in cotyledonary axis): fig. 17, vessels in intercotyledonary plane *B-B*, meristematic cells with denser contents in diagonal planes upper *C* and *D-D*; fig. 18, petiole of first foliage leaf distinct from epicotyl.  $\times 70$ .

level the meristematic ring of the epicotyl changes from an oval (fig. 16) to an ellipse (fig. 17), which is oriented with its long axis at right angles to the cotyledonary plane.

The uppermost portion of the 16-day-old seedling shows the differentiation of the first two leaves, and the structure of the epicotyl indicates where the third leaf will develop.

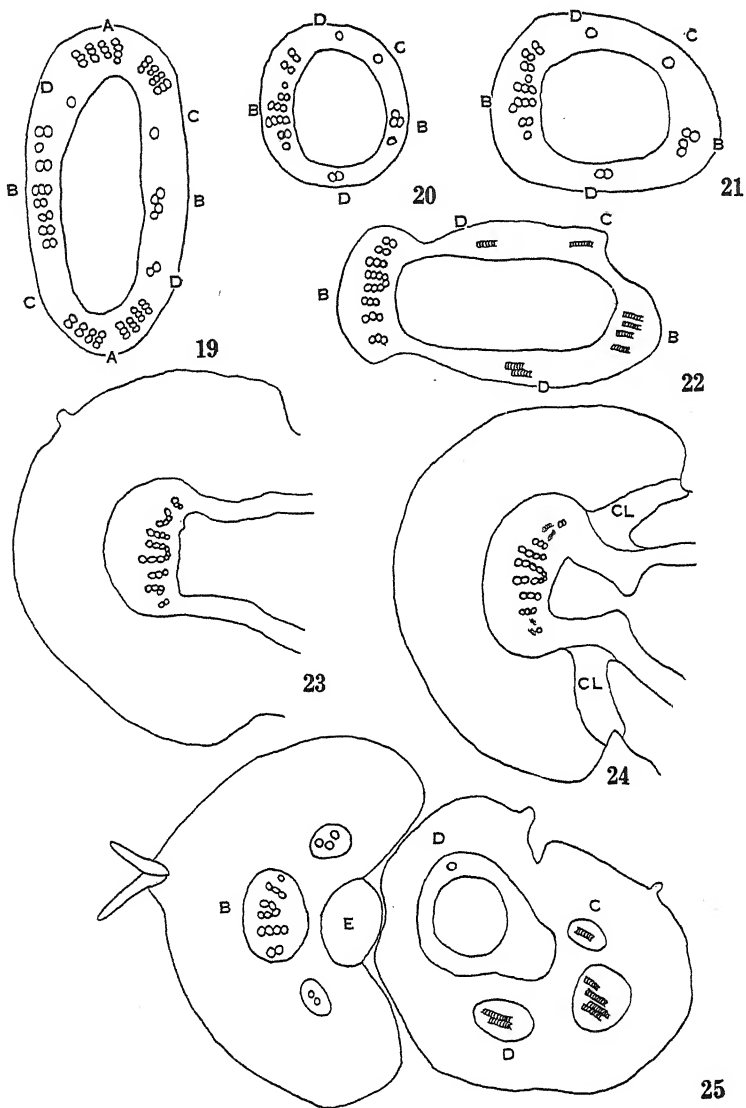
The vessels which are continuous with the first foliage leaf trace are shown in lower *B*, figure 17. The one or two vessels opposite this xylem arc in the intercotyledonary plane are continuous with the midrib of the second foliage leaf (upper *B*, fig. 17). The protuberant tissue of the epicotyl, which later diverges as the petiole to the first leaf, is visible (*B*, fig. 18) as it was in the younger seedling. At the level where the first leaf is diverged from the epicotyl, two conspicuous groups of cells occur (*C* and upper *D*, fig. 18) in the meristematic ring farthest from the petiole. These are continuous with the laterals of the second foliage leaf.

#### DEVELOPMENT OF LEAF TRACES

When the seedling is 24 days old the origin, development, and termination of the first foliar elements may be followed.

At the cotyledonary node xylem elements differentiate in the intercotyledonary plane (fig. 19, *B-B*) and are directly continuous with the elements of the hypocotyl. These vessels in the intercotyledonary plane are more numerous on one side than on the other, and this condition is correlated with the phyllotaxy. At the cotyledonary node one xylem vessel occurs on one side of the cotyledonary arcs of vessels (upper *C* and *D*, fig. 19) while on the opposite side two vessels are differentiated (lower *D*, fig. 19). The meristem of the epicotyl is continuous and oval in shape as in the younger seedlings (fig. 20). In this ring, which becomes more and more elliptical at successively higher levels, there are now four vessels (*B*, fig. 21) instead of two as shown in figure 18. Regions *D* and *C* each have one vessel, and lower *D* has two instead of the meristematic groups that occurred in the 16-day seedling.

At this level the arc of vessels (fig. 22) that is continuous with the first leaf is oriented in transectional view, while the vessels that are continuous with the traces of the second and third leaves appear in



FIGS. 19-25.—Figs. 19-22, diagrams showing meristematic ring and vessels of 24-day-old seedling (A-A, cotyledonary plane; B-B, intercotyledonary plane; C-C and D-D, diagonal planes): fig. 19, section at hypocotyl showing cotyledonary vessels A-A, which become traces of first and second foliage leaves B-B, and vessels in diagonal planes C and D-D; figs. 20-21, vessels of epicotyl; fig. 22, vessels which become traces of first foliage leaf differentiating in vertical plane, opposite them the vessels which become traces of midrib of second foliage differentiating in horizontal plane and adjacent vessels also differentiating in horizontal plane. Figs. 23, 24, transverse sections through petiole of first leaf; fig. 23, vessels intact; fig. 24, a few vessels on either side of intact group; CL, region where cleavage will take place. Fig. 25, transverse section of 24-day-old plant showing petiole of first foliage leaf distinct with midrib and two laterals; vessels in diagonal planes C-D differentiating to form laterals to second leaf; meristematic ring complete with vessel D which is continuous with midrib of petiole of third leaf.

longisection. The groups of vessels situated at each end of the arc (*B*, figs. 23, 24, 25) form the laterals of the first leaf.

The four vessels (right *B*, fig. 22) and the adjacent vessels (*C* and lower *D*, fig. 22) are continuous with midrib and lateral traces of the second leaf (fig. 25). The protuberance of the epicotyl (fig. 25) contains three groups of vessels and at a higher level there is a cleavage line in the parenchymatous tissue where the petiole of the second leaf is diverged. In the ring of meristematic tissue is seen one vessel (upper *D*, fig. 25) which is one of those continuous with the midrib of the third leaf.

### Discussion

A paper by SILER (5) thoroughly reviews the history of the work on root-stem transition. This paper stresses CHAUVEAUD's (2) theory in contrast to that of VAN TIEGHEM (9). The transition stages observed in *Hibiscus trionum* are in agreement with CHAUVEAUD's theory.

The type of transition found in *Hibiscus trionum* differed from that found in *Thespesia* by SINNOTT (6). SINNOTT's diagrams indicate that at the cotyledonary node there are two collateral bundles in each cotyledonary petiole, and six bundles in the region of the epicotyl. The bundles of the cotyledonary petiole vary from two at the point of petiolar attachment to four at the base of the cotyledonary blade.

*H. trionum* deviated from this method. From the typical radial, tetrarch root, two phloem groups appeared between each of the four xylem arcs. At the cotyledonary node there were four xylem groups in each cotyledonary petiole. These were formed from the cotyledonary xylem appearing as two groups owing to the obliteration of the protoxylem elements of the cotyledonary poles and from the division of the xylem rays of the intercotyledonary plane. This indicates that the entire primary vascular system of the root is continuous with that of the cotyledons. After the divergence of the cotyledonary petioles, the lateral groups of meristem in the intercotyledonary plane become continuous. This process is repeated after the separation of each succeeding petiole. These meristematic cylinders occurring repeatedly are procambial tissue.

SINNOTT (6) states, "By far the most common condition at the cotyledonary node is that shown in *Thespesia populnea*, *Momordica balsamina*, and *Lavatera arborea* where the bundles of the epicotyl arise entirely in the intercotyledonary plane and the traces of each cotyledon make but a single gap in the vascular ring." He further states that this corresponds to the unilacunar nodal type in the mature stem. *H. trionum* might also be added to this list, because of its conformity in regard to the bundles of the epicotyl arising in the intercotyledonary plane as well as to the fact that the traces of each cotyledon make but a single gap in the vascular cylinder.

The "double bundle" found in the petiole of the cotyledons of *H. trionum* is comparable with THOMAS' (7) second type of "double bundle." She states, "it was found that the 'double bundle' is sometimes represented by two quite separate bundles (*Ricinus*, *Quercus*, etc.). . . . The function of these central bundles, whether they appear as two widely separate entities, or so closely approximated as to have the appearance of a single strand, is always the same, viz., to form between them one pole of the root." She also says, "in addition to the two central bundles or their 'double bundle' equivalent, there is frequently present a lateral bundle on each side of them, so that there are four bundles in each cotyledon (*Ricinus*, *Quercus*, *Fagus*) or the equivalent of four (*Casuarina*, *Polygonatum*, *Crataegus*, *Acacia*, *Medicago*, *Althaea*, etc.)." As *Althaea* is one of the Malvaceae and a close relative of *Hibiscus*, this correlation between the two is to be expected.

THOMAS (7) also states, "when the lateral bundles are large and important looking, they penetrate into the hypocotyl and unite with the corresponding bundle from the opposite cotyledon to form the alternating poles of a tetrarch root." This is precisely the condition found and carried out in the root-stem transition of *H. trionum*.

### Summary

1. The anatomy and transition stages from root through hypocotyl of 5-day-old seedlings of *Hibiscus trionum* were studied; the vascular anatomy of the epicotyl of 16-day-old and 24-day-old plants was also examined.
2. The differentiation of "intermediary" vessels, and the disap-



pearance of some protoxylem elements in the root and hypocotyl, cause each of the four radial xylem rays to appear in the shape of an arc.

3. Obliteration of the protoxylem elements and further differentiation in the hypocotyledonary region result in the rearrangement of the vessels of each arc into two more or less distinct groups.

4. The intercotyledonary plane is made up of active meristematic cells in the 5-day-old seedling.

5. The xylem in the cotyledonary plane is formed from the polar xylem group and a part of each of the lateral groups.

6. The cotyledonary trace is made up of a "double bundle" and two laterals.

7. The vessels that differentiate in the intercotyledonary plane of the 16- and 24-day-old seedlings form the traces to the first and second leaves which occur at alternate nodes. Only one element of the third leaf had been differentiated in the 24-day-old seedling.

8. The vessels constituting the two arcs in the intercotyledonary plane vary greatly in number.

9. The lateral vessels in the petiole of the first leaf are formed from the intact arc of vessels in that petiole.

10. The lateral vessels in the petiole of the second leaf arise in a longitudinal diagonal plane in the hypocotyl region.

11. The cotyledonary node is elliptical in the cotyledonary plane while the second and third nodes are elliptical in the opposite plane. The fourth and fifth nodes are elliptical again in a plane above and parallel with the cotyledonary plane.

12. The recurring meristematic ring is procambium.

I wish to express my gratitude to Dr. EMMA N. ANDERSEN whose assistance has made this work possible and to Dr. ELDA R. WALKER for many helpful suggestions.

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## DEVELOPMENT OF THE MACROGAMETOPHYTE IN CERTAIN LABIATAE

ELIZABETH P. BUSHNELL

(WITH TWENTY-TWO FIGURES)

### Introduction

SCHNARF (4, 5) has summarized the observations upon the formation of the nucellus, integument, archesporium, and macrospores in the Labiatae. The ovule is anatropous, with a single thick integument. The archesporial cell of the nucellus is subepidermal; it functions as a macrospore mother cell. Of the four macrospores formed by its division, the innermost develops into a seven-celled, eight-nucleate gametophyte. The antipodal cells disintegrate in most cases before fertilization. The polar nuclei fuse in the chalazal part of the gametophyte. Endosperm formation follows one of four somewhat diverse types in the various genera.

RUTTLE (2, 3) has traced the history of the macrogametophytes of *Meniha* and *Lycopus*. Minor differences occur in species of *Mentha* and *Lycopus* in size of macrospores, time of development of the tapetal layer, shape of gametophyte, time of fusion of the polar nuclei, and the presence or absence of an obturator.

In *Monarda fistulosa* and *Nepeta cataria*, BILLINGS (1) describes a large coenocytic micropylar haustorium containing a few relatively small nuclei.

### Material and methods

Development of the macrogametophyte has been studied in *Monarda fistulosa* L., *M. didyma* L., *M. punctata* L., and *Nepeta cataria* L. Most of the material used was collected in the Pharmaceutical Gardens of the University of Wisconsin; some of the material of *M. punctata* came from plants growing in sandy fields near Pine Bluff, Wisconsin, and some of *M. didyma* from private gardens in the vicinity of Madison.

Flower buds and individual flowers were fixed in Karpechenko's, Navashin's, Licent's, Flemming's medium, and Carnoy's acetic-

alcohol-chloroform solutions. Best results were obtained with Nava-shin's, Licent's, and Flemming's fluids. Fixed material after the usual treatment was imbedded in paraffin. Sections were cut from 10 to 15  $\mu$  in thickness and stained with Flemming's triple stain or with Heidenhain's iron-alum haematoxylin.

All figures were drawn with a camera lucida at table level. A Leitz microscope with a  $\frac{1}{16}$  inch, 1.32 N.A. oil immersion objective, and 3 $\times$ , 4 $\times$ , 8 $\times$ , and 12 $\times$  oculars was used.

### Observations

#### *Monarda fistulosa*

When microspore mother cells are first recognizable in the anther, the ovule (one in each ovary lobe) appears as a slight protuberance from the base of the sporangial chamber. It curves as it grows (fig. 1), finally becoming anatropous. The nucellus consists of one layer of cells.

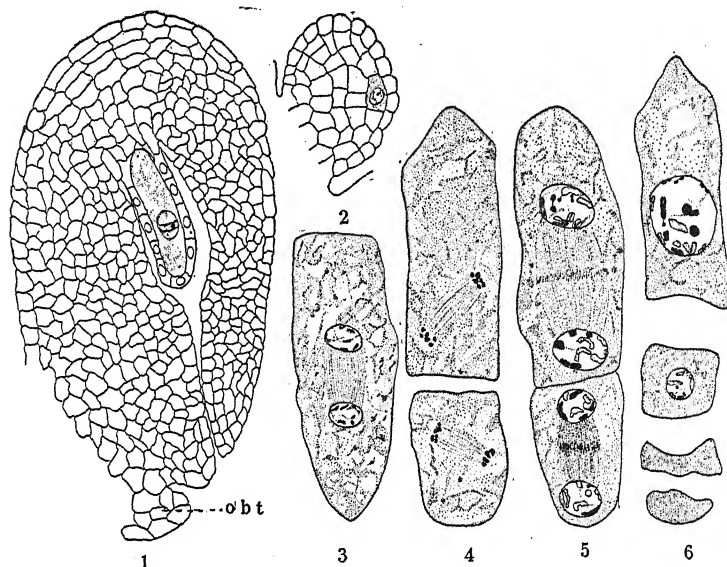
An archesporial cell, differentiated before the single integument shows much indication of development (fig. 2), functions directly as a macrospore mother cell. In some instances the integument has surrounded the nucellus when the nucleus of the macrospore mother cell is still in a presynizetic stage. Macrospore mother cells with nuclei in synizesis, however, were observed in other ovules whose integument incloses the nucellus by the time of formation of the heterotypic equatorial plate.

When the single integument has grown about half way around the nucellus, a projection (the obturator, fig. 1) appears below the ovule. When the ovule is fully developed, the massive obturator extends around the micropyle.

Two nuclear and cell divisions (figs. 3, 4) result in the formation of a typical row of four macrospores. Both cell divisions are brought about by means of cell plates (fig. 5). Degeneration of the macrospores is invariably from the micropyle backward; the large spore at the chalazal end is the one that functions (fig. 6). This macrospore enlarges and large vacuoles appear above and below the nucleus. After nuclear division, the two daughter nuclei lie in the central region of the macrogametophyte; however, they soon move apart (fig. 7) and the macrogametophyte elongates and broadens. A large

vacuole forms in the central part, smaller vacuoles now being present at the ends. The large vacuole also characterizes the four-nucleate stage (fig. 8), but is no longer present at the eight-nucleate stage (fig. 9).

The mature seven-celled gametophyte is broad at its micropylar end; the remainder is narrow and tubelike (fig. 10), curving some-

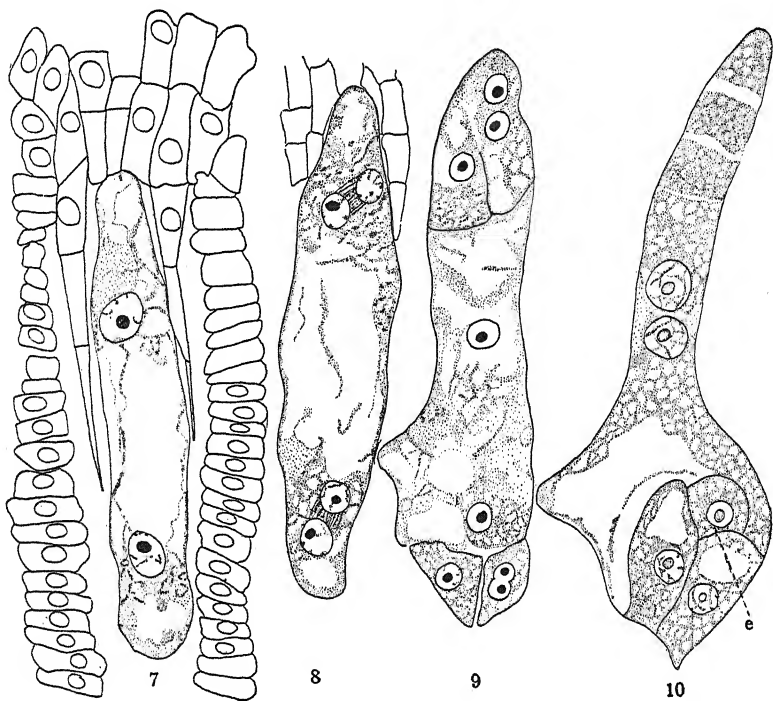


FIGS. 1-6.—Macrogametophyte development in *Monarda fistulosa*: Fig. 1, ovule showing single integument and obturator (*obt*); macrospore mother cell surrounded by one-layered nucellus. Fig. 2, young ovule showing archesporial cell. Fig. 3, telophase, heterotypic division. Fig. 4, anaphase, homoeotypic division. Fig. 5, cell-plate formation following homoeotypic division. Fig. 6, chalazal macrospore persisting, other three macrospores disintegrating. Figs. 1, 2,  $\times 315$ ; all others,  $\times 1025$ .

what toward the vascular bundle of the ovule. The egg, as it grows, becomes differentiated into a stalk, in contact with the macrogametophyte wall, which contains little stainable cytoplasm, and an enlarged spherical portion with rather densely staining cytoplasm. The synergids are long and narrow. On the sides nearest the periphery of the macrogametophyte each synergid develops a ridge. The effect of a partial cap is produced, as described by RUTTLE (2) for *Mentha requienii*. The antipodals may sometimes be seen disinte-

grating before the two polar nuclei have fused. Fusion of these nuclei occurs before fertilization in the central part of the cell.

At the period of the heterotypic diakinesis, nucellar cells nearest the micropyle appear somewhat crushed and stain darkly. Lateral nucellar cells at the two-nucleate stage of the macrogametophyte

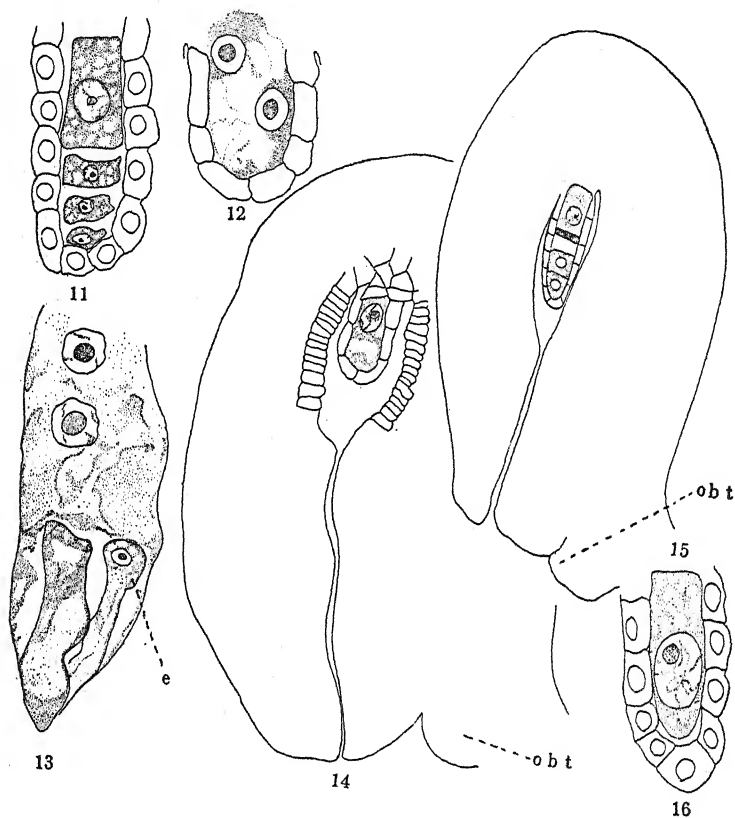


FIGS. 7-10.—Macrogametophyte development in *Monarda fistulosa*: Fig. 7, two-nucleate gametophyte, a few chalazal nucellar cells, "tapetum," and hypostase. Fig. 8, four-nucleate macrogametophyte. Fig. 9, eight-nucleate macrogametophyte, cell division in progress. Fig. 10, macrogametophyte showing egg (*e*), synergids, and two polar nuclei; antipodal cells already disintegrated.  $\times 785$ .

now also take a dark stain and begin to disintegrate. As the two-nucleate gametophyte grows, it pushes out through the nucellar epithelium and crushed nucellar cells are then digested. Only a few cells in the chalazal region of the nucellus persist during the entire period of development of the macrogametophyte.

Cells in the chalazal portion of the integument become modified

to form the hypostase tissue. At the time when there is an axial row of macrospores, the hypostase consists of only a few chalazal cells which stain darkly. When the gametophyte is one-nucleate, the walls of the hypostase cells are thickened. The hypostase is well de-

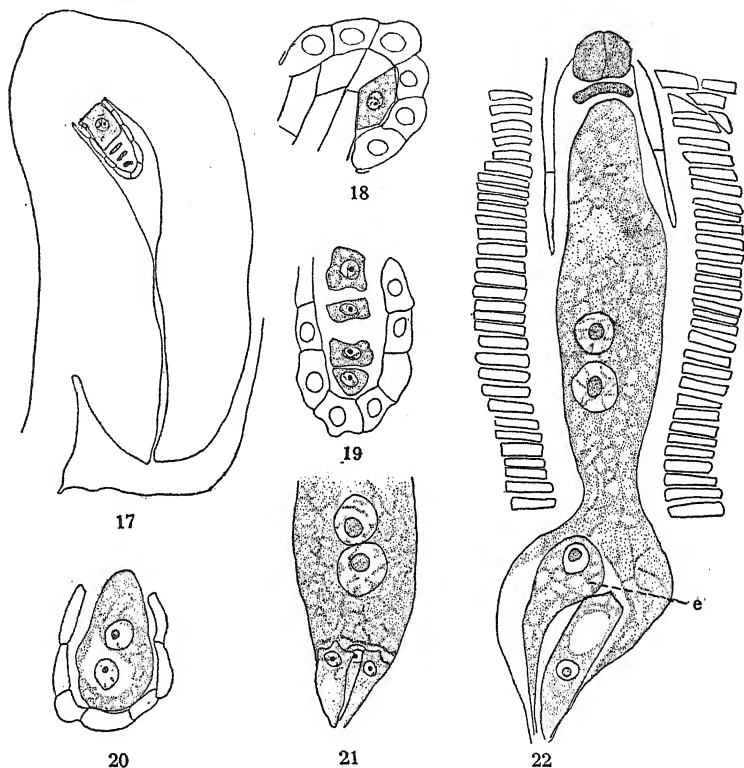


FIGS. 11-16.—Macrogametophyte development in *Monarda didyma* (figs. 11-14) and in *M. punctata* (figs. 15, 16): Fig. 11, four macrospores, the chalazal the largest. Fig. 12, nucellus with two-nucleate macrogametophyte. Fig. 13, egg (*e*), one synergid, and polar nuclei. Fig. 14, ovule with massive integument, large micropylar cavity, obturator (*obt*), macrospore. Fig. 15, ovule showing integument, obturator, and four macrospores. Fig. 16, macrospore mother cell. Figs. 14, 15,  $\times 250$ ; all others,  $\times 625$ .

veloped at the two-nucleate stage and remains prominent throughout the further development of the macrogametophyte.

As a result of the nucellar disintegration, the innermost "tapetal"

layer of the integument comes to lie next to the gametophyte. At the four-nucleate stage the cells of this layer appear regularly arranged and have dense cytoplasmic contents.



FIGS. 17-22.—Macrogametophyte development in *Nepeta cataria*: Fig. 17, ovule showing large micropylar cavity, functional chalazal macrospore, and three disintegrating spores. Fig. 18, young ovule containing archesporial cell. Fig. 19, four macrospores. Fig. 20, two-nucleate gametophyte and nucellar layer. Fig. 21, egg apparatus and polar nuclei. Fig. 22, egg (*e*), one synergid, polar nuclei, disintegrating antipodals, nucellar cells, and "tapetum." Fig. 17,  $\times 250$ ; all others,  $\times 625$ .

### *Monarda didyma*

Macrospore formation and development of the macrogametophyte proceed as in *M. fistulosa*. The four macrospores are smaller and slenderer than those of *M. fistulosa* (fig. 11). At the two-nucleate stage the macrogametophyte differs in shape from that of *M.*



*fistulosa*, being broad and not greatly elongated (fig. 12). The size of the mature gametophyte corresponds with that in *M. fistulosa*, but the micropylar portion is less broad (fig. 13).

The integument in *M. didyma* is larger than in *M. fistulosa* and the micropyle is longer. The hypostase is not so conspicuous as in *M. fistulosa*, nor is the obturator so large (fig. 14).

#### *Monarda punctata*

Development of the macrogametophyte is essentially as in *M. fistulosa* and *M. didyma* (figs. 15, 16). The integument does not surround the nucellus so early as in *M. fistulosa*, but incloses it by the time the macrospores are formed. The obturator is smaller than that of *M. fistulosa*, about the same size as in *M. didyma*. The hypostase is not so conspicuous as in *M. fistulosa* or in *M. didyma*.

#### *Nepeta cataria*

Development of the macrogametophyte proceeds as in the three species of *Monarda* (figs. 17-22). The integument is somewhat massive. When it finally surrounds the nucellus, a large micropylar cavity remains between the nucellus and the integument. No obturator is formed.

#### Summary

1. Development of the ovule and macrogametophyte are similar, except for minor differences, in *Monarda fistulosa*, *M. didyma*, *M. punctata*, and *Nepeta cataria*. The ovule is anatropous, has a single massive integument, a one-layered nucellus which does not persist, a hypostase tissue, and an integumentary "tapetum."
2. The chalazal macrospore is the functional one and the embryo sac is of the common seven-celled type. *M. fistulosa* has the largest macrospores.
3. An obturator is developed in all but *N. cataria*.
4. The integument incloses the nucellus later in *M. punctata* than in the other species. It is thicker in *M. didyma* and *M. punctata* than in *M. fistulosa* and *N. cataria*.
5. The hypostase is very prominent in *M. fistulosa*, rather prominent in *M. didyma* and *N. cataria*, and not so conspicuous in *M. punctata*.

I am indebted to Dr. C. E. ALLEN for helpful suggestions and kindly criticism throughout the course of the study.

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## BRIEFER ARTICLE

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### TECHNIQUE FOR PREPARING MICROSCOPIC SECTIONS OF WOODY STEMS AND ROOTS

In the autumn of 1934 the writers desired to make microscopic sections of the stems and roots of hard woody desert trees and shrubs. Their method is similar to that used in the preparation of slides of petrifications. Sections 2-3 inches in diameter, of uniform thickness, and in many cases as thin as 10  $\mu$ , were cut. These could be examined with an oil immersion lens and photomicrographs made from them. A brief description of the method follows.

PREPARATION OF SECTIONS.—A section 2-4 mm. thick was cut in the desired plane, the surfaces being parallel. Sections larger than 2 inches were difficult to handle. One surface of the section was then smoothed by first placing coarse sandpaper on a glass plate and rubbing the section on it. After the saw marks were removed, finer grades of sandpaper were used, and the section was finally polished on the finest grade of emery paper. The section so prepared was mounted on a glass slide with Canada balsam. The mounted section was now dressed down to the desired thickness with fine sandpaper and emery paper. The final grinding was done on clean worn emery paper with a very light pressure applied on the strokes. When the section was sufficiently thin, it was removed from the slide with xylene. Following the washing away of grit and balsam with xylene, the section was remounted in fresh balsam of ordinary consistency.

STAINING.—Several different methods of staining were tested. The most satisfactory results were secured by placing the sections, after being cut, in alcoholic stains for a period of a few hours to a day or more, depending upon the hardness of the wood. A few good double-stained slides were made by placing the sections in the counter stain and leaving them there until the stain had had time to penetrate the wood, after which they were dried and dressed down in the manner just described. Some good slides were made by staining with iodine green and orange G. The stain which gave the sharpest differentiation of structures with most woods was made by dissolving 2 gm. of acid fuchsin in 100 cc. of a saturated alcoholic solution of picric acid.

Silver nitrate has certain effects upon woody tissues which may make

it a valuable stain. The following method was used. A section was ground down to about  $20\ \mu$  in thickness, and then covered with a few drops of 2 per cent solution of silver nitrate. This was allowed to stand two or three minutes and was then washed with distilled water, after which it was exposed to a strong light until it turned brown. It was then air dried and ground to the final thickness and mounted without removal from the slide. In only a few cases were the sections uniformly impregnated with silver, but in nearly all the slides there were areas which showed a particularly sharp differentiation of tissues.

The most satisfactory slides were made without staining. In nearly all of the unstained sections the natural coloring of the wood and the difference in the refractive index of the various structures were sufficient to assure a well defined differentiation of tissues. While this method of sectioning has been found to be laborious, the results have been satisfactory. Sections of soft wood, less than 1 inch in diameter, have been prepared in 20 to 30 minutes, but larger sections of hardwoods have taken as long as four hours.—W. W. NEWBY and PERRY PLUMMER, *University of Utah*.

## CURRENT LITERATURE

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*The Morphology of Vascular Plants, Lower Groups (Psilophytales to Filicales).*

By ARTHUR J. EAMES. New York: McGraw-Hill Company, 1936. Pp. 433. Figs. 215 and frontispiece.

The author states in the preface that this book is not intended as a reference work but as a textbook. "The viewpoint is that of broad comparative study with the development of a natural classification and phylogenetic relationships as a goal." The life history and type method of presentation of the older morphological works is discarded and the material for the consideration of morphology from the comparative and phylogenetic standpoints is presented in an order and form more consonant with the ideals of the new morphology. Important new material which has accumulated from recent work, not only on the living but on the fossil lower vascular plants, is included. The descriptive text is concisely arranged under a regular sequence of headings, with the pertinent illustrations conveniently inserted at the appropriate places. These excellent illustrations were all prepared by Mrs. EAMES, and the many original ones she herself drew. They and the original photographs by Mr. W. R. FISHER add greatly to the value of the volume. Each chapter is followed by a brief discussion and summary. There is an excellent 21-page index, so classified and complete as to make comparison of important individual features an easy matter. Such an index makes the book very useful for comparative studies, while the excellence of the print and the almost complete absence of typographical errors add to its attractiveness.

Although the subject matter is treated fundamentally from the phylogenetic standpoint, the sequence in which the orders are considered is not phylogenetic but that found most satisfactory from the teaching standpoint. This leads naturally to the consideration of the living forms first, as an introduction to that of the fossil. The last chapter deals with theoretical views on the organization of the plant body and the discussion of the bearing of the results from living and fossil forms on classification.

The chapters which gave the reviewer most pleasure were X and XI, dealing with the water ferns. The biological features of the two families which are ordinarily included in this group are marshalled in a particularly masterly way and convincing evidence brought forward to show that this is not a natural group, but that the Marsiliaceae have come from Gleichenian stock while the Salviniaceae are more closely related to the Hymenophyllaceae. Then too the proposed classification of the vascular plants (Tracheophyta) into four

groups, the Psilopsida, Lycopsida, Sphenopsida, and Pteropsida, represents a decided step toward a natural grouping, the old classification into Pteridophyta and Spermatophyta having a tendency to obscure the connection of the modern seed plants (gymnosperms and angiosperms) with the ferns. One misses, however, the term Verticillatae in this classification. It would seem more appropriate for the horsetails and their fossil allies than Sphenopsida since it emphasizes the distinctly radial structure not only of the sporophyte but of the gametophyte, and sets this group apart from the others in a very decided way, indicating that they probably have been derived from stock more closely related to the bryophytes than have the other forms of the lower vascular plants. Although this would break up the uniformity of the terminology of the classification, it would seem to be warranted by the distinctiveness of the group. EAMES' view of the origin of the sporangiophore also shows how much importance he attaches to this verticillate character.

There are a few features which the reviewer would have liked to have seen treated differently. These are concerned mainly with embryogeny. D'ARCY THOMPSON has shown that the shape and arrangement of the quadrant and octant cells of the animal embryo conform with the requirements of the laws of minimum surface. That these laws are equally applicable to the corresponding stages of plant embryos, as well as to those of gametophytes, pollen grains, etc., is self-evident. Although EAMES has recognized the impossibility of the application of the old quadrant-organography theory in many cases, he still considers that it holds in some, particularly in leptosporangiate ferns. For example, he states of *Marsilia* (p. 220): "Quadrant segments are formed (Fig. 142E), and these bear definite relation to the first organs; the two outer segments form leaf and root, the inner, stem and foot (Fig. 142F)"; and of *Azolla* (pp. 254-5): "Definite quadrants are formed which develop the four primary organs, leaf, stem, root, and foot, as is typical for leptosporangiate ferns (Fig. 166E)." The first difficulty here is that the quadrants of *Marsilia* and other leptosporangiate ferns may be formed by two longitudinal divisions, instead of one longitudinal and one transverse as postulated by the quadrant theory. In this case each quadrant cell would represent parts of two organs, for example, part of the root and part of the foot. Even if it be considered that organ segregation is delayed until the octant stage, a complex change in the mechanism of organ segregation would be involved, and its postulation would be of slight avail since at a later stage the cells from one sector reinforce those from another in forming the first adult organs. The problem is certainly a much more complicated one than the quadrant-organography theory can explain. A further difficulty has to do with one of the postulated four quadrant organs, the so-called foot. The embryo in its early stages absorbs food through all its peripheral cells. Later a specialized area of contact for absorption (an haustorial region) may be formed. This is not necessarily located at the base of the archegonial axis as in bryophytes,

so that the term foot, which implies such location, is inappropriate. It does occur, however, always in connection with the bulk of the food material of the prothallium, whether it appears on the proembryo (before the adult organs are formed) or on some part of an adult organ, as for example at the base of the first leaf whorl in *Equisetum* or at the base of the first leaves in Marattious ferns. BOWER in his new book, *Primitive Land Plants*, has fittingly designated such haustoria "opportunistic growths"! It certainly should not be given equal rank with true organs as is implied by the quadrant theory. On the other hand EAMES' relegation of the root to the place of an adventitious organ (p. 163, etc.) does not seem to be in keeping with the prominence which he gives it in the quadrant. Much could be said on this point but it would involve a consideration of sporeling organization and its relationship to that of the adult, and this aspect has not been included in the present work. It can only be said that the root physiologically and in origin is the equivalent of the shoot and that their course of evolution is of equal importance. It therefore beclouds the problem to call the primitive type of root in such forms as the Psilotaceae by the term rhizome. It was one of the great contributions of the late Dr. D. H. SCOTT that he recognized this and by the use of inverted commas around his terms "root" and "shoot" provided botanists with means of readily designating the primitive type of subterranean and subaerial organs of the plant. There is another aspect of embryogeny to which it seems appropriate to refer here. EAMES, in striking contrast to BOWER, regards the suspensor as of "little or no morphological significance" (p. 297) and even discounts its physiological function. While not attempting to reconcile these opposing views, certain important features may be pointed out. Depth of location of the cells from which the embryo proper develops permits an advanced formation of its adult organs while still within the prothallium, thus allowing the plant to get a good start in life. The original depth of the egg is thus important and the deepening of embryogeny by a suspensor equally so, whether its cells actively elongate and "push" or are passive.

There is one disadvantage which is inherent in the very nature of the textbook type of presentation: the evidence for the opinions expressed cannot always be included and the reader is at a loss as to the basis upon which they are founded. It would be gratifying to know upon what evidence the statement on p. 351 that heterospory "had led to the seed habit" is founded. There are also some statements on the character of the tapetum which it would be interesting to be able to trace to their origin.

The foregoing are minor criticisms, however, in view of the general excellence of the book and the value it will have in revivifying interest in an important field which has been neglected in recent years because of lack of a suitable text.—R. B. THOMSON.

*Quantity of Living Plant Materials in Prairie Soils in Relation to Runoff and Soil Erosion.* By J. E. WEAVER and G. W. HARMON. Bull. 8, Conservation Dept. of Conservation and Survey Division, Univ. of Nebraska. 1935.

*Comparison of Runoff and Erosion in Prairie, Pasture, and Cultivated Land.* By J. E. WEAVER and WM. C. NOLL. Bull. 11, Conservation Dept. of Conservation and Survey Division, Univ. of Nebraska. 1935.

*Relative Efficiency of Roots and Tops of Plants in Protecting the Soil from Erosion.* By JOSEPH KRAMER and J. E. WEAVER. Bull. 12, Conservation Dept. of Conservation and Survey Division, Univ. of Nebraska. 1936.

In view of the importance of the problem of soil erosion and of the recent increase in the activities for combating this national menace, attention should be called to the studies of WEAVER and his students and co-workers. These investigations, carried on at Lincoln, Nebraska, supply needed data of a quantitative nature for certain aspects of the effect of vegetation in impeding soil erosion. Further studies of this type are urgently needed in various parts of the country so that recommendations in regard to soil conservation methods may be placed completely on a basis of fact.

The three bulletins deal with different aspects of the same general theme. In the first the emphasis is upon the consistent decreases in the amounts of underground plant materials found in a series of soil samples taken from relatively undisturbed prairie types, and those secured from the early, medial, and late stages in the deterioration and degeneration of the prairie caused by overgrazing. "On uplands decreases in dry weight were 35, 40, and 72 per cent respectively, from the original sod in the surface 4 inches. . . . On lowlands decreases of 28, 40, and 77 per cent were found in the surface layer."

The second bulletin reports the results of a study of the amounts of runoff and erosion on prairie, pasture, and cultivated land by the runoff-plot method, in which rainfall was supplemented by artificial watering. "Runoff on a 10° slope from 26.88 inches of rainfall during 15 months was 2.5 per cent from prairie, 9.1 from overgrazed pasture, and 15.1 per cent from a pasture entirely bared by close grazing. The soil was Carrington silt loam. No measurable amount of soil eroded from the prairie, only a small amount from the pasture, but 5.08 tons per acre were lost from the bare area." On April 13, 1935, 2.5 inches of water were applied to these plots at the rate of 2 inches per hour. "Runoff losses were 0, 29.3, and 50.4 per cent, respectively, and losses by erosion 0, 165 lbs., and 3.42 tons of soil per acre. . . . Runoff on a 5° slope from 12.9 inches of rainfall during a period of 11 months was 1 per cent from prairie, 12.1 per cent from wheat field, and 17.8 per cent from fallow land. . . . No measurable erosion occurred in prairie, 0.52 ton of soil per acre eroded from the wheat field, and 2.6 tons from the fallow land." Other experiments no less striking bear out the conclusions that "a soil covered with its natural mantle of climax vegetation represents conditions most favorable to maximum absorp-



tion of rainfall and maximum erosion control," that "soils that have been depleted of their organic matter and are poor in structure are less absorptive and are easily eroded," and that "one of the chief essentials of erosion control is the increased use of grasses."

The third bulletin is a detailed report of the relative efficiency of roots and tops acting together and underground parts alone in protecting soil from erosion. Numerous field and garden crops, pasture crops, weeds, and native grasses were tested, and the efficiency was determined by the length of time required for the complete removal of the soil in a sample 1 meter long, 0.5 m. wide, and 1 dm. deep by artificial water erosion under controlled conditions. The samples were secured in the field, so that the soil structure and vegetation were undisturbed. In all cases, as one would expect, the resistance to erosion was much greater when the above-ground plant parts were not removed. In general, the importance of these parts seems to be related to the degree and character of cover by which they prevent the direct impact of falling water upon the bare soil surface. "Prevention of erosion does not result so much from vertical thickness of cover as from one widely spread and continuous." In crop plants "the effect with plant cover intact exceeds that of underground parts alone 3 to 7 times. . . . Close spacing of rows or broadcasting would result in greatly increased soil protection. Any practice which removes plant cover or debris is undesirable from the viewpoint of soil conservation. . . . All weeds are of some value as soil conservers. . . . Remaining on the ground after maturity, they function during life and after death." Space does not allow further introduction of the data or conclusions; anyone seriously interested in the problem of soil erosion should consult these reports.—C. E. OLMSTED.

*A Popular Guide to the Higher Fungi (Mushrooms) of New York State.* By LOUIS C. C. KRIEGER. New York State Museum Handbook 11, Albany, N.Y.: The University of the State of New York, 1935. Pp. 538 with 126 text figures and 32 colored plates. \$2.00.

Few books can compare with this handbook in vivid expression combined with scientific accuracy and high quality of illustrations. It is a responsible presentation by one who is an expert, not alone in faithfully delineating form and color, but in presenting each illustration as a composition. Chapters include an instructive account of fairy rings with a list of the species commonly forming them; a discussion of the seasonal occurrence of certain genera and species, with a tabular summary; habitat relations of important species, particularly with regard to New York State, ably summarized. There is a chapter on the collection and preparation of mushrooms for the herbarium that should be valuable for the beginner and also afford a standard for the serious student of mycology. Another chapter contains a number of recipes for preparing mushrooms for the table—desiderata not to be ignored in a book of this character. A chapter on

their cultivation is a condensed description of usual practice. A discussion of the poisonous mushrooms of the state deals with the more dangerous species and ready means for distinguishing them, symptoms produced by them and the requisite treatments. A chapter on wood-destroying fungi, although containing much interesting information, may seem somewhat extraneous, even though some of the species are mushrooms. The literature of the subject includes extensive citations arranged according to genera. Then follows the systematic account, over half of the book, containing a chart showing the classification of important genera accompanied by pertinent line drawings, a workable key to orders, families, and genera, a discussion of taxonomic characters and their terminology, followed by specific descriptions. These latter employ the least possible technical verbiage without loss of accuracy. It must be borne in mind that throughout there are references to some 600 additional related species, so that the work is actually more comprehensive than is indicated by the 213 species especially treated.

Completing the book are an excellent bibliography, a carefully selected glossary of technical terms, a set of 32 colored plates, and a well elaborated index. There are few typographical errors and the volume is a credit to the institution sponsoring it. It is a landmark among publications on fungi, and will be useful both to serious students and to those whose interest is chiefly gastronomic.

It is unfortunate that at this time another printing of this work, at a higher price and ostensibly covering a greater field but differing only in some title modifications and a few deletions, including the omission of a map, has been issued by a commercial concern and credited to the same author, although apparently without his knowledge. A general handbook of fleshy fungi for a larger area would properly have included a much greater number of KRIEGER's beautifully executed paintings of mushrooms which have not yet been published and which are not available to the general public.—W. W. DIEHL.

*Lehrbuch der Botanik für Hochschulen, begründet von Strasburger, E.; Noll, F., Schenck, H., und Schimper, A. F. W.* By FITTING, HANS; HARDER, RICHARD; SIERP, HERMANN; KARSTEN, GEORGE. Jena: Gustav Fischer, 1936. Pp. xii+628. figs. 868.

For many years STRASBURGER had been planning the comprehensive textbook of botany which made its first appearance in 1894. At that time, Drs. NOLL, SCHENCK, and SCHIMPER were associated with him in the famous laboratory at Bonn. STRASBURGER wrote the morphology; NOLL, the physiology; SCHENCK, the bryophytes and pteridophytes; and SCHIMPER, the spermatophytes. The plan and scope of each part was discussed before the writing; and afterward, by repeated conferences, duplication and overlapping were eliminated. That the plan was good and the work well done are proved by the fact that, in spite of changing authorship, this is the nineteenth edition. There were

five editions before the death of SCHIMPER; STRASBURGER wrote eleven editions; NOLL, nine; and SCHENCK, sixteen. Consequently the fundamental ideas of the book were thoroughly established. KARSTEN, who was also associated with STRASBURGER in the Bonn laboratory, took SCHIMPER's place and has written thirteen editions. In the physiology, NOLL wrote nine editions; JOST, six; while for SIERP, this is the second and for HARDER, who followed SCHENCK, it is also the second.

The new book is a thorough revision, with most of the former material retained but with differences in viewpoint. HARDER recognizes alternation of generations in *Cladophora*, *Ulva*, and *Enteromorpha*; but in *Valonia*, where reduction of chromosomes takes place in the formation of gametes, he says one generation is suppressed. In the Phaeophyceae he recognizes an alternation of generations in forms like *Ectocarpus*, *Cutleria*, and the Laminariaceae, but not in the Fucaceae. He reaches the conclusions which one is likely to reach if he assumes that x generation is synonymous with gametophyte and 2x generation with sporophyte. Often they are synonymous, but in cases like *Ulothrix* and *Fucus*, where they are not synonymous, there is confusion. Below the level of sexuality there is only one generation, the x generation. The 2x generation arose by the fusion of gametes; and from this point the history of alternation is to be traced in the evolution of the new 2x generation, and the reduction of the x generation. He finds alternation in the red algae but in many cases does not homologize it with alternation in the greens and browns. Alternation is recognized in fungi like *Puccinia*, although the reviewer would not agree with the limits set for the two generations. In the higher groups, where x generation and gametophyte and 2x generation and sporophyte are synonymous, there is not much difference of opinion, since the old mistaken idea of homologous alternation went into discard.

Aside from some differences of opinion, largely in respect to alternation of generations, the treatment of the thallophytes, bryophytes, and pteridophytes is excellent and brings this subject up to date. HARDER concludes that the origin of the Archegoniates is entirely unknown.

KARSTEN's part, the seed plants, has not been changed so much in content, the principal changes being in point of view and in the perspective which comes from long and intimate association with the subject. KARSTEN's earlier researches, especially in thallophytes and gymnosperms, were the beginning of the firsthand knowledge which makes his part of the book so reliable.

To make room for some increase in the amount of material, many of the figures have been made rather small, but they are clear and easily understood. The numerous life history diagrams, with the gametophytic phase in light lines and the sporophyte in coarse lines, while not always in accord with my own theories of alternation of generations, are clear and sum up the subject at a glance. Throughout the work, references are abundant and up to date.—C. J. CHAMBERLAIN.

- A. Engler's *Syllabus der Pflanzenfamilien. Eine Übersicht über das gesamte Pflanzensystem mit besonderer Berücksichtigung der Medizinal- und Nutzpflanzen nebst einer Übersicht über die Florenreiche und Florengebiete der Erde zum Gebrauch bei Vorlesungen und Studien über spezielle und medizinisch-pharmazeutische Botanik. Elfte ergänzte Auflage.* By LUDWIG DIELS. Berlin: Gebrüder Borntraeger, 1936. Pp. xliii+419. Figs. 476.

The eleventh revised edition of ENGLER's classical *Syllabus der Pflanzenfamilien* by DIELS brings this standard reference work up to date, twelve years having elapsed since the preceding edition appeared. In size it is almost exactly the same as the ENGLER-GILG edition of 1924, being, however, provided with a few more illustrations. A comparison of the two editions shows changes here and there throughout the text, bringing the work into conformity with developments of the past twelve years. The major groups remain essentially the same, even to the families, although segregates have been recognized in some cases, and some wholly new families are admitted. Perhaps most of the changes have been occasioned by the desire to bring this summary into conformity with the second edition of *Die natürlichen Pflanzenfamilien* as far as that work has appeared. The advances and changes that have been made are more striking when one compares the clearly printed edition of 1936 (the edition of 1924 suffers badly by comparison) with the original edition of 1892, which consisted of 207 pages without illustrations. In form the eleventh edition closely approximates its predecessors. This work is an indispensable aid to the working botanist, no matter what his field and whether a teacher of the subject or an investigator, as it provides a concise summary of the salient features of the entire plant kingdom following ENGLER's modification of the EICHLER system.—E. D. MERRILL.



# THE BOTANICAL GAZETTE

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## LIMITED GROWTH AND ABNORMALITIES IN EXCISED CORN ROOT TIPS<sup>1</sup>

WILLIAM J. ROBBINS AND VIRGINIA B. WHITE

(WITH SIX FIGURES)

### Introduction

In earlier reports (8, 9, 10, 11) from this laboratory, attention has been called to the limited growth of excised corn root tips in a sterile modified Pfeffer's solution containing 2 per cent dextrose, and to the beneficial effects of the addition of peptone or of autolyzed yeast to this medium. Associated with the limited growth is the development of abnormalities in the roots. Aside from the condition of the roots at the time of their transfer to the culture flasks and the environmental conditions other than the culture medium itself, the obvious explanation for the limited growth and the development of abnormalities in the modified Pfeffer's solution may be placed in one of two categories. Either the medium is inadequate in one or more respects or it is injurious because of the presence or development of some injurious condition. In the present paper a further study of the limited growth and development of abnormalities in excised corn root tips under sterile conditions is reported.

We have investigated as possible causal factors the water used in preparing the medium, the carbohydrate used, the mineral salts, hydrogen-ion concentration, aeration, and the effect of certain additions to the medium. The experiments suggest a deficiency in the medium as the probable cause for the results.

<sup>1</sup> Supported in part by a Grant in Aid from the National Research Council.

### Materials and methods

The methods used were essentially those reported earlier. Grains of Longfellow flint corn<sup>2</sup> were sterilized with calcium hypochlorite and germinated in petri dishes on a 0.5 per cent water agar. After germination, the main root tip was severed by a sharp knife and transferred to the sterile medium in which it was grown. Usually a single root tip was grown in a culture vessel.

The solutions used were a modified Pfeffer's solution previously described; a simpler and more dilute solution (solution C) prepared to imitate more closely the soil solution; and solution C to which 0.1 p.p.m. of manganese chloride, 0.1 p.p.m. of zinc chloride, and 0.1 p.p.m. of sodium borate were added. The last solution is referred to as solution CF. Solution C contained 50 p.p.m. calcium nitrate, 10 p.p.m. magnesium sulphate, 10 p.p.m. potassium dihydrogen phosphate, 1 p.p.m. ferric chloride, and 2 per cent dextrose. The dextrose unless otherwise noted was a preparation of high purity made by Coleman and Bell and labeled "Special for Injection." Culture vessels of pyrex glass were used throughout. The water employed was twice redistilled from pyrex glass.

In most of the experiments the roots were grown in 30 cc. of medium in 125 cc. Erlenmeyer flasks, and kept in the dark at room temperature without change of culture medium or transfer of the root tips to fresh medium.

### Experimental results

GROWTH IN SOLUTION C.—In solution C, prepared with the Coleman and Bell dextrose, the growth of excised corn root tips was distinctly limited. The average final length of 225 tips originally 1 cm. long, in sixteen experiments performed at different times, was 8.6 cm. with an average of 39 secondary roots per root. The minimum length of a single root in these experiments was 4.0 cm. with no secondary roots, and the maximum was 44.1 cm. with 191 secondary roots. The smallest average growth in a single experiment was 5.6 cm. with an average per root of 22 secondary roots and the maximum was 15.1 cm. and 75 secondary roots.

<sup>2</sup> Thanks are due Dr. J. K. WILSON of Cornell University, who kindly supplied the corn used throughout these investigations.

Associated with this limited growth was the development of abnormalities, the most common of which was a water-soaked and swollen condition. In the development of this abnormality the root sinks to the bottom of the flask and the region of elongation becomes

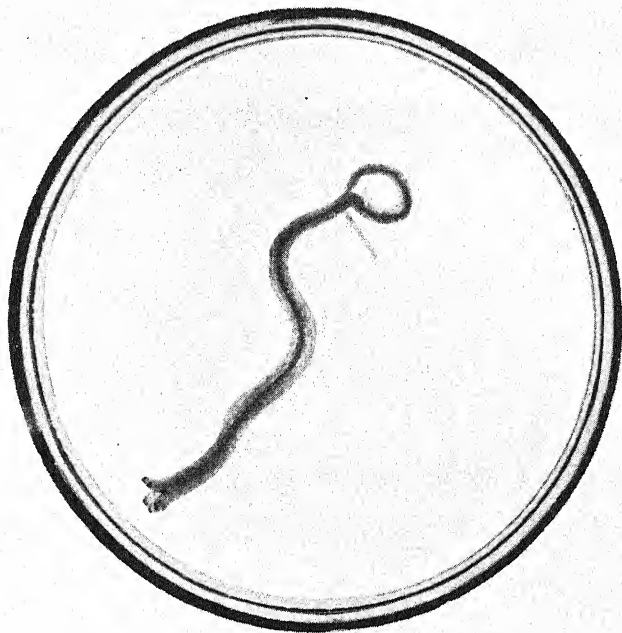


FIG. 1.—Abnormal root after two weeks in solution C; original length 1 cm. Note swelling and the stele which is clearly visible because of water-soaked condition of root. Splitting at base is also evident.

translucent, the apical meristem standing out plainly as a whitish area due to the displacement by liquid of the gas in the intercellular spaces. The water-soaked portion, at first limited to the region of elongation, later extends backward toward the basal end of the root into the region of differentiation, and as a result the stele shows plainly through the cortex (fig. 1). Associated with the water-



soaked condition is a swelling of the cortex, increasing the diameter of the root several times in the more extreme cases and sometimes becoming so pronounced at the basal end that the cortex splits away from the central cylinder. This may occur also near the tip. Such abnormal roots grow slowly and produce branches which may not extend beyond the swollen cortex or which develop into short swollen branches or into longer secondaries with short stubby branches. The abnormal roots are stiff and friable and eventually become yellowish or brownish in color.

The first evidences of this abnormal condition may appear within a day after transfer of the roots to solution C or they may be delayed until some later stage in the growth of the root. There is considerable variation also between individual roots in the same experiment, some becoming abnormal in a day or two and others remaining normal or nearly normal for the duration of the experiment (figs. 2, 3).

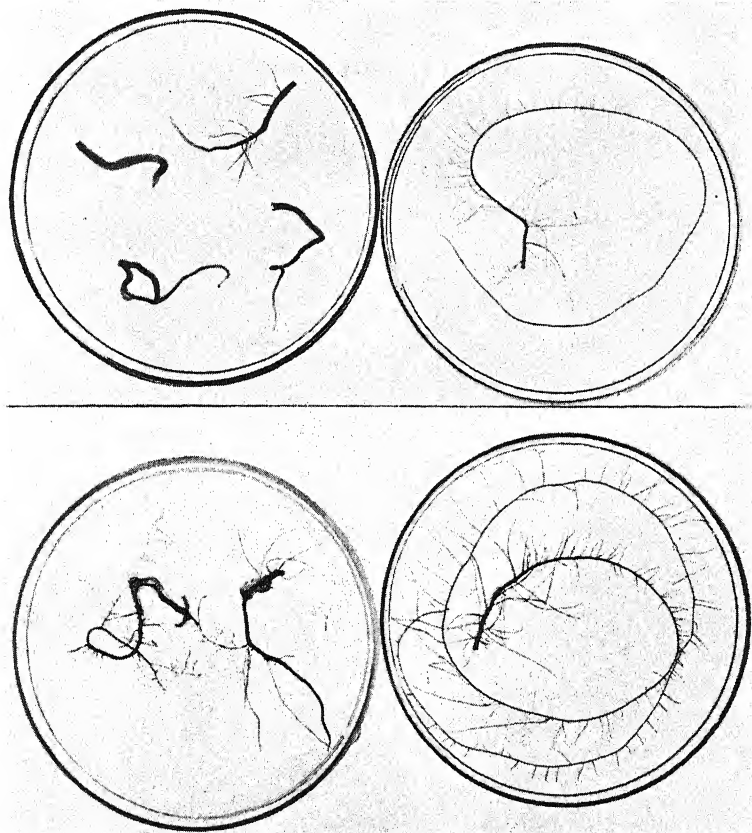
Another type of abnormality observed during the first few days of growth is a slight swelling of the region of elongation on which long root hairs had developed. The tip of the root became black and the cortex in the region of abundant root hairs swelled, wrinkled, and sometimes split away from the central cylinder. The heavy production of root hairs near the tip gave such roots a dry and woody appearance.

The proportion of roots developing such abnormalities varied from experiment to experiment. At the end of ten days or two weeks abnormal roots made up from 10 to 100 per cent of those cultured in solution C, the modified Pfeffer's solution and similar solutions.

What is the cause for these results? Is it (1) the distilled water used in preparing the medium, (2) the dextrose, (3) the mineral salts, (4) the hydrogen-ion concentration, or some other factor or combination of factors?

1. DISTILLED WATER.—It is well known that the purity of the distilled water may be an important factor in determining growth in culture solutions. In the present experiments, however, the presence of contaminants is not considered to be responsible for the limited growth and development of abnormalities in solution C. The water used was redistilled from a special still constructed of pyrex glass. The distilled water which was redistilled came from a block

tin-lined Tripure still. In some cases a small amount of barium hydroxide was added before redistillation. In other cases a trace of sulphuric acid or of sulphuric acid and potassium permanganate



FIGS. 2, 3.—Fig. 2 (above), normal root and abnormal roots from same experiment; original length 1 cm.; grown two weeks in solution C. Fig. 3 (below), normal and abnormal roots grown in solution C after pretreatment on agar or filter paper; original length 1 cm.; grown two weeks at room temperature in the dark. Left, abnormal roots (note swollen and split cortex); right, normal root.

was added before redistillation and the distillate again distilled from a barium hydroxide solution, making a triply distilled water. Triply distilled water was prepared also by distillation from acid permanganate and from a permanganate solution made alkaline with

potassium hydroxide. Little difference was found in the growth of excised corn root tips in solution C prepared with these various kinds of distilled water.

2. CARBOHYDRATE.—Next to water, dextrose (customarily used at 2 per cent concentration) comprises more of solution C than any other ingredient. It is desirable to determine, therefore, whether the kind of carbohydrate, its purity, or change in its constitution as the result of sterilization may be responsible for the results secured in solution C or similar solutions.

ROBBINS (8) reported dextrose to be superior to levulose as a carbon source for excised corn root tips grown in a modified Pfeffer's solution. WHITE (18) states that sucrose was more satisfactory than dextrose for the growth of excised tomato roots in the solution he used. The writers have compared dextrose, levulose, xylose, sucrose, and maltose as carbon sources for excised corn root tips. The results show dextrose to be somewhat superior to levulose or sucrose and very much better than either xylose or maltose.

The brands of sugars used in making the comparisons were: dextrose, anhydrous (Eastman Kodak Company); levulose, for diabetics (C.P., Pfanstiehl); sucrose (standard sample no. 17, Bureau of Standards); maltose, purified malt sugar (Merck); xylose (Eastman Kodak Company).

Excised corn root tips of 0.2 cm. original length were grown individually in 125 cc. Erlenmeyer flasks containing 30 cc. of solution C plus 0.5 per cent agar and 2 per cent dextrose, 2 per cent levulose, or 1.66 per cent cane sugar. The dextrose and levulose were added to the medium before sterilization; the sucrose was separately sterilized in redistilled water and added to the agar and mineral salts which had also been separately sterilized. The cultures were incubated 84 days in the dark at room temperature. The results given in table I show dextrose to be somewhat superior to levulose or sucrose.

Root tips of approximately 2 cm. original length were grown in the modified Pfeffer's solution containing 2 per cent dextrose or 2 per cent cane sugar. Here also somewhat better growth was found in the solution containing dextrose.

Root tips of 1 cm. original length were grown individually in

125 cc. Erlenmeyer flasks containing 50 cc. of the modified Pfeffer's solution made up with 1 per cent dextrose, 1 per cent xylose, or a mixture of 1 per cent xylose and 1 per cent dextrose. The cultures were incubated in the dark for 81 days. The xylose was used by the roots to some extent but was less satisfactory than the dextrose and at this concentration appeared somewhat injurious. The roots in the dextrose solution grew more than twice the length of those in the xylose solution. The growth in the mixture of xylose and dextrose was superior to that in the xylose alone but much inferior to that in the dextrose alone.

TABLE I

GROWTH OF EXCISED CORN ROOT TIPS IN SOLUTION C PLUS 0.5 PER CENT AGAR WITH DEXTROSE, LEVULOSE, OR SUCROSE AS SOURCE OF CARBON. ORIGINAL LENGTH 2 MM. GROWN 84 DAYS IN DARK AT ROOM TEMPERATURE

CARBOHYDRATE	No. ROOTS	AVERAGE FINAL LENGTH (CM.)	AVERAGE NO. SEC- ONDARY ROOTS	MAXIMUM LENGTH (CM.)	MAXIMUM NO. SEC- ONDARY ROOTS	MINIMUM LENGTH (CM.)	MINIMUM NO. SEC- ONDARY ROOTS
Dextrose.....	18	16.7	61	31.5	117	4.5	12
Levulose.....	17	11.9	33	23.5	77	5.5	1
Sucrose.....	15	12.2	21	26.0	72	3.3	1

The effectiveness of maltose as compared with dextrose was determined by growing root tips of 1 mm. original length in 30 cc. of a medium composed of solution CF plus 0.5 per cent agar and 2 per cent dextrose or maltose. Two root tips were grown in each flask and the cultures were incubated for 52 days in the dark at room temperature. The results summarized in table II show the inadequacy of maltose as a source of carbon for excised corn roots. The average growth in the maltose series was about one-fourth of that in the dextrose series. The roots in the maltose series became very thin, and if they attained a length of more than 3 cm. the tips had the diameter of a thin thread and broke easily when moved.

KNUDSON (6) grew entire plants in the light in the presence of various carbohydrates and found maltose less satisfactory than dextrose. The differences he found, however, were not so marked as those reported here, probably because the plants were supported in part by carbohydrates from the seed or grain and from photo-

synthesis. An excised root tip is limited almost completely to the carbohydrate supplied by the medium.

It is surprising that maltose should prove so much less satisfactory than dextrose. Maltose yields dextrose on hydrolysis and a considerable part of the food in the corn grain is starch which is hydrolyzed to maltose by amylase. It may be that the corn root does not produce maltase. It is possible also that the differences in growth result in part from contaminants in the sugars used. Sugars are difficult to secure free from mineral elements and nitrogenous substances, and these samples without doubt contained some foreign material.

TABLE II

GROWTH OF EXCISED CORN ROOT TIPS IN SOLUTION CF PLUS 0.5 PER CENT AGAR AND 2 PER CENT DEXTROSE OR MALTOSE. ORIGINAL LENGTH 1 MM.  
GROWN 52 DAYS IN DARK AT ROOM TEMPERATURE

CARBOHYDRATE	NO. ROOTS	AVERAGE FINAL LENGTH (CM.)	AVERAGE NO. SECONDARY ROOTS	MAXIMUM LENGTH (CM.)	MAXIMUM NO. SECONDARY ROOTS	MINIMUM LENGTH (CM.)	MINIMUM NO. SECONDARY ROOTS
Dextrose.....	20	10.3	31	26.1	77	3.1	4
Maltose.....	16	2.8	1	6.0	5	0.5	0

In any event the experiments confirm the earlier conclusions that dextrose is a better carbohydrate for the growth of excised corn root tips than other sugars which might reasonably be considered and which can readily be secured.

Considerable differences have been observed in the effects of different brands of dextrose, however, and even different lots of the same brand, on the growth of excised corn root tips in solution C. This is illustrated by the following experiment in which seven different brands of dextrose were compared in solution C.

In this case root tips of 1 cm. original length were grown individually in 125 cc. Erlenmeyer flasks containing 30 cc. of solution C. The roots were grown 49 days at room temperature in the dark. Seven different samples of dextrose were used. Three times as much growth was secured in the culture solutions prepared with sample no. 1 as with sample no. 7 (table III).

We are not prepared to state the reason for the differences in the growth secured with the different brands of dextrose. Is it something organic or inorganic, something beneficial or detrimental? For example, is the difference between the effects of sample no. 1 (which gave the best results) and sample no. 7 (which gave the poorest) due to something beneficial in no. 1 or something detrimental in no. 7. These samples are both of high purity. The hydrogen-ion concentration of solution C prepared with these two samples was the same. Neither contained appreciable amounts of iron or of

TABLE III

GROWTH OF EXCISED CORN ROOT TIPS IN SOLUTION C PREPARED WITH DIFFERENT BRANDS OF DEXTROSE. ORIGINAL LENGTH 1 CM. GROWN 49 DAYS IN DARK AT ROOM TEMPERATURE

BRAND OF DEXTROSE	GRADE	NO. OF ROOTS	AV. FINAL LENGTH (CM.)	AV. NO. SECONDARY ROOTS	MAXIMUM LENGTH (CM.)	MAXIMUM NO. SECONDARY ROOTS	MINIMUM LENGTH (CM.)	MINIMUM NO. SECONDARY ROOTS
1	C. P., special for injection.....	15	24.5	111	64.4	300	6.5	18
2	Granular.....	14	11.5	33	25.8	109	5.8	4
3	C.P.....	15	10.8	57	22.2	178	5.6	20
4	U.S.P.X.....	15	10.5	60	21.5	140	5.6	23
5	Analytical reagent	14	12.7	82	17.9	159	8.1	29
6	C.P.....	14	19.5	68	69.5	326	8.0	21
7	Special for injection.....	13	8.8	43	16.2	115	5.7	19

copper. The ash content of no. 1 was 0.008 per cent, somewhat greater than that of no. 7 which contained 0.003 per cent ash. This might suggest the presence of beneficial ash elements in no. 1 as the responsible factor, particularly as the ash content of sample no. 6 which gave good results was 0.034 per cent and that of no. 3 which gave poor results was 0.003 per cent. On the other hand, the ash of a 2 per cent solution of no. 1 is but 1.6 p.p.m. and the amount of ash added in the dextrose to 30 cc. of medium (the quantity customarily used for culturing a single root tip in these experiments) would be 0.048 mg. Any single element would make up but a fraction of this amount. It is not beyond the bounds of possibility, however, that the differences in the effects of the different brands of

highly purified dextrose are associated with the presence or absence of traces of the rarer elements. In any event the results are significant in emphasizing the importance of the character of the dextrose used in culture media. While minute attention may be devoted to the water or the mineral salts employed in culturing microorganisms, fungi, and other living things, investigators may pay little attention to the dextrose used, provided it is of high grade. It would be desirable to perform further experiments in which dextrose completely free of nitrogen and ash elements is used.

There remains the question as to whether sterilization of the solutions produced substances from the carbohydrate which were injurious to the roots. It is well known that dextrose is decomposed by heating in alkaline solutions, with the development of furfural, organic acids, and other products. SMITH (14) found that phosphates accelerated the decomposition. CONDREA and ROTH (2) and CIANCI and PANNAIN (1) found some decomposition occurred and some acid developed in the sterilization of dextrose in distilled water. The writers have confirmed this observation and also found that acid was formed from dextrose sterilized intermittently at 100° C. in a nutrient solution initially acid to neutrality.

It is not believed, however, that the limited growth and development of abnormalities in solution C are the result of decomposition products formed from the dextrose in sterilization. Comparisons of the growth of excised root tips in solution C prepared by sterilizing the dextrose and mineral salts separately and together at 100° C. on three successive days showed no significant differences. Further, the growth of roots was similar in solution C containing dextrose sterilized by heating at 100° C. and by filtration through a porcelain candle. This observation would seem to eliminate products formed from dextrose in sterilization as a factor of importance in determining the growth of excised roots in our experiments.

3. MINERAL SALTS.—The mineral salts used in solution C are significant from the standpoint of their purity, concentration, proportions, and adequacy.

The salts used in preparing the mineral nutrient solutions were of the customary chemically pure grade. In preparing solution C the following were used:  $\text{Ca}(\text{NO}_3)_2$  (Merck White Label),  $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$

(Merck Reagent),  $\text{KH}_2\text{PO}_4$  (Merck Reagent),  $\text{FeCl}_3$  (Merck Reagent).

It is not thought that any contaminant in these chemicals is responsible for the poor growth of root tips in solution C. The growth in solution C prepared with the stock chemicals was compared with that in a similar solution made from salts purified by recrystallization.<sup>3</sup> Little difference was found in the effects of the two types of solutions.

The concentration of salts in solution C is low, totaling 70 p.p.m. It is not likely, however, that this is responsible for the results secured. No difference was found with the modified Pfeffer's solution which contains 642 p.p.m. of salts nor with solution C at five times (350 p.p.m.) its normal concentration. The concentration of dextrose remained constant at 2 per cent.

No experiments were performed in which the proportion of the various salts included in solution C was systematically varied, although it is recognized that proper balance is an important requirement in any nutrient solution. However, various nutrient solutions have been used (see section following on other nutrient solutions) and at one time or another the proportions of salts have been varied, particularly the ratio of calcium to magnesium, without material effect on the resulting growth. It is doubtful whether the salt proportions in solution C are of first importance, although such a statement cannot be made categorically without more extensive and systematic experiments.

The adequacy of the mineral salts in solution C should be considered particularly from the standpoint of the supply of iron and of various micro-essential elements.

In solution C iron is furnished as ferric chloride at a concentration of 1 p.p.m. Whether iron in this form and at this concentration is adequate may well be asked. WHITE (17) reports that ferric sulphate at 2.5 p.p.m. is more satisfactory than ferric chloride or ferric citrate.

While not prepared to answer the question finally at this writing, we do not think that the iron supply is the limiting factor in these experiments. We have substituted ferric tartrate and ferric citrate

<sup>3</sup> The purified chemicals were kindly prepared by Dr. ALBERT E. SAEGER.



for ferric chloride without marked effect, and have found additions to the medium (filter paper and water extract of filter paper) which add less than 0.2 p.p.m. of iron to improve growth decidedly.

An excised corn root tip is more limited in its stored mineral elements than is a root attached to a corn grain from which new supplies of ash elements may be translocated into the tip. It might be anticipated that a deficiency of one or more of the essential elements would be evidenced in the growth of excised root tips more readily than in the growth of roots attached to the grain. Although it was recognized that the chemicals used in preparing solution C contain traces of other elements as contaminants, there is still the possibility that the limited growth in that solution and similar solutions is the result of a deficiency in one or more of the rarer mineral elements.

Experiments were performed in which salts of manganese, boron, zinc, copper, and thallium were added to solution C. The growth of roots 1 cm. long in solution CF containing 0.1 p.p.m. each of manganese chloride, zinc chloride, and sodium borate was in some cases definitely better than in solution C. The improvement was not so great, however, as that secured by the addition of agar or of qualitative filter paper (see later paragraph). Root tips 1 mm. in length grew longer and produced more secondary roots in solution CF containing 0.5 per cent agar than in solution C containing agar.

The addition to solution CF of 0.001 p.p.m. or 0.01 p.p.m. of copper sulphate caused no improvement. Since a small decrease in growth was found with the addition of 0.01 p.p.m., it is not believed that further increases in the concentration of copper would prove beneficial. The addition to solution C of thallium nitrate at a concentration of 0.005 p.p.m. was without effect, although RICHARDS (7) has reported thallium beneficial to the growth of yeast.

While these experiments show some benefit from the addition of salts of manganese, boron, and zinc, they do not suggest that their addition to solution C would make it entirely satisfactory for the growth of excised corn roots. It is possible of course that the proper concentrations were not used. More extensive experiments with the rarer elements would be advisable, employing such mixtures as have been used by HOAGLAND and CHANDLER (4) and by TRELEASE and TRELEASE (15).

4. **HYDROGEN-ION CONCENTRATION.**—The hydrogen-ion concentration of solution C and of the modified Pfeffer's solution is near pH 4.5. The additions to Pfeffer's solution and to solution C which were found to be beneficial, such as agar, filter paper, yeast, peptone, or soluble starch (see later), make these solutions somewhat less acid although rarely changing the pH to a value higher than 5.4. There is therefore the possibility that the poor growth in solution C and in the modified Pfeffer's solution is due to too great an acidity. However, results of experiments in which excised corn root tips were grown in these solutions made less acid do not support such a hypothesis.

The modified Pfeffer's solution was adjusted to pH 5.0, 5.6, 5.9, and 6.1 by the addition of KOH, and excised root tips were grown for ten days in the solution. At the end of that time the tips were severed and transferred to fresh solutions of the same reactions. After two weeks the process was repeated. Little difference was noted in the growth of the root tips in the solutions of original pH 4.4, 5.0, and 5.6, although some reduction of growth was found at pH 5.9 and 6.1.

A repetition of this experiment in which potassium nitrate was omitted from the Pfeffer's solution and the concentration of salts was reduced showed no benefit in the less acid solutions. In this case the original reactions of the solutions were pH 4.4, 5.5, 6.8, and 7.1. The root tips in the two less acid solutions grew less well than those in the two more acid solutions.

The reaction of solution C was adjusted by the addition of dibasic potassium phosphate to acidities ranging from pH 4.5 to 5.2. Excised corn root tips 1 cm. long were grown individually in 30 cc. quantities of the medium in 125 cc. Erlenmeyer flasks at room temperature in the dark. In one experiment the salts and dextrose were sterilized together; in a repetition of it the salts and the dextrose were sterilized separately and mixed after sterilization. No significant differences were found in the growth at the different reactions, and none between the cultures in which the dextrose and salts were sterilized separately and those in which they were sterilized together.

That the hydrogen-ion concentration is not the cause of the limited growth in the modified Pfeffer's solution or in solution C was concluded from these experiments.

#### AERATION

In a liquid medium the oxygen available to the excised roots is that which dissolves in the medium, at least as soon as the roots sink below the surface of the liquid. Furthermore, the heating of the liquid in sterilization drives off all dissolved gases and, since the root tips were customarily placed in the culture flasks within a day or two after the sterilization of the medium, it is possible that the gases dissolved in the liquid were not in equilibrium with those of the air. The character of the commonest abnormality observed in the growth of the root tips suggests some interference with respiration, since the water-soaked condition is the result of the displacement by liquid of the gases normally present in the root.

Earlier experiments (11) in which excised root tips were grown in the light in the modified Pfeffer's solution through which a continuous current of air was bubbled did not suggest that the limited growth was due to a deficiency of oxygen. Additional experiments have been performed which bear upon this question. The results show that an increase in the oxygen supply over that secured in a liquid medium improves growth. They did not, however, demonstrate that the eventual stoppage of growth and the development of abnormalities can be entirely prevented by such means as were used to increase the aeration.

In the numerous experiments performed on the growth of excised root tips in liquid cultures, efforts were made to increase the oxygen supply to the root tip by tilting the culture flask in such a way as to drain the liquid from the root tip and expose it to the moist atmosphere in the flask. In other cases the root tip was pulled out of the liquid by a sterile transfer needle and left exposed to the air against the side of the flask. Tips were treated in these ways when they were observed to have become water-soaked and translucent and to have ceased or nearly ceased growth. In both methods the root tip was left exposed to the air until it was no longer translucent or until it had partially dried, before it was again immersed in the

solution. These treatments were sometimes but not always effective in causing the root to lose its water-soaked appearance and to renew its growth.

In order to investigate this systematically, an experiment was performed in which the flasks were tilted and the medium drained from the root tips at regular intervals. Roots were cut 2 mm. long and left for 24 hours on plates containing solution C plus 0.6 per cent agar. They were then grown individually in 30 cc. of solution C or solution C plus 400 p.p.m. of autolyzed yeast in 500 cc. Erlenmeyer flasks for 100 days at room temperature in the dark. For the first

TABLE IV

GROWTH OF EXCISED ROOT TIPS IN SOLUTION C OR IN SOLUTION C PLUS 400 P.P.M. AUTOLYZED YEAST IN 500 CC. ERLENMEYER FLASKS. ONE SET OF FLASKS TILTED TO EXPOSE ROOTS TO AIR AT REGULAR INTERVALS. ORIGINAL LENGTH 2 MM.

MEDIUM	TREAT- MENT	No. ROOTS	AV. FI- NAL LENGTH (CM.)	AV. NO. SECOND- ARY ROOTS	MAXI- MUM LENGTH (CM.)	MAXI- MUM NO. SEC- ONDARY ROOTS	MINIMUM LENGTH (CM.)	MINIMUM NO. SEC- ONDARY ROOTS
Solution C.....	{None	15	5.4	30	8.5	36	3.7	7
	{Tilted	12	11.5	47	45.6	194	5.4	8
Solution C. plus 400 p.p.m. au- tolyzed yeast.	{None	12	7.6	30	19.2	70	4.2	5
	{Tilted	13	12.0	44	24.6	87	5.0	20

30 days of the experiment (by the end of this time most of the roots had ceased growth) some of the flasks were tilted and the liquid drained from the root tip for approximately eight out of every 48 hours. Some improvement in growth was obtained by this treatment (table IV). The development of abnormalities in both solution C and solution C plus yeast was delayed and reduced by the tilting, although some water-soaked and swollen roots developed in the tilted series.

Some benefit, therefore, was derived from thus regularly exposing the roots to the air, but of course such treatment also removed them for the time being from the medium. To whatever the improvement of the roots was due, the treatment did not permit more than limited growth and did not eliminate the abnormalities.

It was thought that the depth of the liquid in the flasks might be of some importance. No significant difference was found, however, between the growth of roots in 30 cc. of liquid in 125 cc. flasks or the same quantity in 500 cc. flasks. No difference was observed between roots grown in 15 cc. and in 30 cc. of medium in the 125 cc. Erlenmeyer flasks. Such differences in oxygen supply as result from varying the depth of the liquid within the limits given above did not influence the growth of the root tips in solution C.

This should not be interpreted as indicating that oxygen supply is not a significant factor in the growth of excised corn root tips. A comparison of roots grown on the surface of an agar medium and

TABLE V

GROWTH OF EXCISED ROOT TIPS IN 30 CC. OF SOLUTION C PLUS 0.5 PER CENT AGAR.  
ORIGINAL LENGTH 1 CM. ROOT TIPS LEFT ON SURFACE OF MEDIUM  
IN ONE CASE; PRESSED TO BOTTOM OF MEDIUM IN OTHER CASE

POSITION	NO. ROOTS	AV. FINAL LENGTH (CM.)	AV. NO. SECOND- ARY ROOTS	MAXIMUM LENGTH (CM.)	MAXIMUM NO. SEC- ONDARY ROOTS	MINIMUM LENGTH (CM.)	MINIMUM NO. SEC- ONDARY ROOTS
Surface.....	10	36.9	184	90.0	295	11.8	77
Below surface....	9	11.8	32	24.7	64	5.0	15

roots which had been pushed to the bottom of the flasks showed a decided difference in favor of the former.

Roots cut 1.0 cm. long were grown individually for 87 days in 30 cc. of solution C containing 0.5 per cent agar in 125 cc. Erlenmeyer flasks at room temperature in the dark. Some of the roots were left as usual on the surface of the agar, others were pressed gently through the medium to the bottom of the flasks. The results summarized in table V show that those roots pressed to the bottom of the flasks grew much less than those originally left on the surface of the agar. The difference in the roots grown on and under the agar is shown also in figure 4. Furthermore, three of the roots which had been pushed to the bottom of the flasks grew so that at some time during the experiment a portion of the root was exposed at the surface of the agar. These roots attained the greatest length and produced the largest number of secondary roots in the series under agar.

Besides the difference in final length and number of secondary roots, there was also a difference in the time at which secondary roots were formed in the two series. By the fourth day secondary roots had been formed by all those placed on the surface of the agar. On the roots which had been pressed to the bottom of the flasks branches developed on one root on the seventh day, on one on the tenth, on one on the thirteenth, on six on the nineteenth, and on one on the twenty-second day.

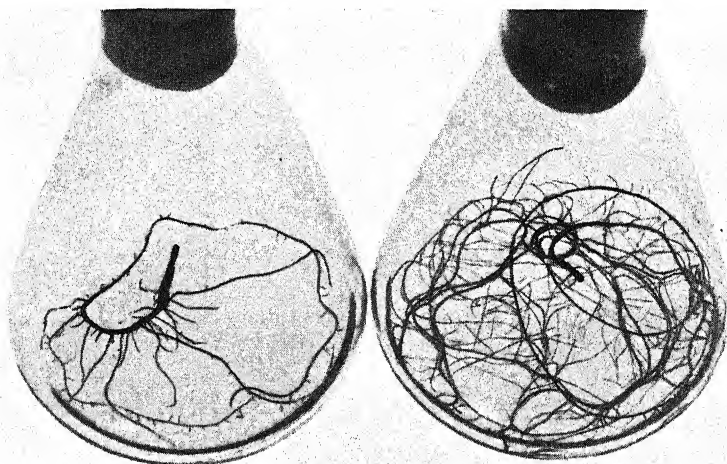


FIG. 4.—Typical roots grown 80 days in solution C plus 0.5% agar; original length 1 cm. Left, root which had been pushed through medium to bottom of flask; right, root left on surface of agar.

The marked difference in the growth of the two sets of roots is somewhat surprising because those originally on the surface soon grew down under the agar and the major portion developed beneath the surface.

Although the oxygen available to those roots grown under the agar was certainly no greater than that available in solution C without agar, the development of abnormalities was much less pronounced. For the first nineteen days no signs of abnormalities aside from a decrease in diameter and a deficiency of branches were observed in the roots grown under the agar. This is in contrast to the pronounced abnormalities which developed in solution C within the first few days of growth. On the nineteenth day a slight swelling

of the cortex at the basal end of some roots was observed. This was followed by a wrinkling of the cortex and the development of a translucent area near the basal end; but the roots under the agar did not develop the water-soaked appearance in the region of elongation of the main root, a condition so characteristic of many roots grown in solution C. The secondary roots were somewhat irregular in length and characteristically somewhat swollen, translucent, and stiff; the entire root systems of the members of the series under agar were slightly discolored. These abnormalities are to some extent the same as those observed in roots grown in solution C, but in the latter they appear earlier and are accentuated.

TABLE VI

GROWTH OF EXCISED CORN ROOTS IN SOLUTION C AND IN SAME SOLUTION PLUS 0.5 PER CENT AGAR. ORIGINAL LENGTH 1 CM. GROWN IN 30 CC. OF MEDIUM IN 125 CC. ERLENMEYER FLASKS FOR 57 DAYS IN DARK AT ROOM TEMPERATURE

MEDIUM	No. ROOTS	AV. FINAL LENGTH (CM.)	AV. NO. SECOND- ARY ROOTS	MAXIMUM LENGTH (CM.)	MAXIMUM NO. SEC- ONDARY ROOTS	MINIMUM LENGTH (CM.)	MINIMUM NO. SEC- ONDARY ROOTS
Solution C.....	17	6.9	22	9.7	41	5.5	3
Solution C + 0.5% agar.....	20	34.2	178	92.0	448	4.0	20

AGAR.—In experiments performed earlier in this laboratory, superior growth of excised corn roots was secured in an agar medium. No attempt at that time was made, however, to compare the growth in a liquid medium with that on agar nor to determine the reasons for the beneficial effects of the agar. The writers have found that the growth of excised corn root tips is markedly improved and the percentage of abnormalities reduced by the addition of Difco agar to solution C.

The superiority of solution C containing sufficient agar to make a semi-solid medium is shown in table VI, in which excised root tips 1 cm. long were grown individually in 30 cc. of medium in 125 cc. Erlenmeyer flasks for 57 days at room temperature in the dark.

The beneficial effects of the agar are due to its water-soluble constituents and not to its colloidal character nor to the semi-solid

condition of the medium. This conclusion follows because a water extract of the agar added to solution C produces the same beneficial effect as the agar itself (fig. 5). The water extract was prepared by extracting 30 gm. of Difco agar twice with 500 cc. of redistilled water for 48 hours in the icebox and adding to solution C an amount of the filtered extract equivalent to 0.5 per cent agar.

No attempt was made to determine whether the beneficial effect of the agar was due to its organic or to its inorganic elements. The water extract of the agar made solution C somewhat less acid, raising the pH from 4.5 to 4.8. From other experiments in which the effect of hydrogen-ion concentration on the growth of excised roots

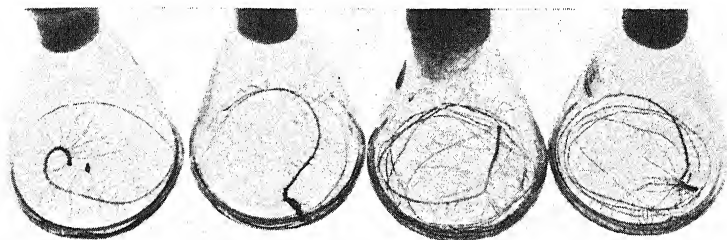


FIG. 5.—Effect of water extract of agar. From left to right: two best roots grown in solution C; two best roots grown in solution C plus water extract of agar. Original length 1 cm.; grown 47 days at room temperature in the dark.

in the modified Pfeffer's solution and in solution C was studied, it is not believed that the effect of the agar or of the water extract of the agar was due to its effect on the reaction of solution C.

**FILTER PAPER.**—Not only was a favorable effect obtained by the addition to solution C of agar or a water extract of agar, but the addition of a qualitative filter paper (Arthur H. Thomas 90 mm. circles no. 5160) proved even more beneficial. The beneficial effects of the filter paper were observed in solution C prepared with purified chemicals, solution C prepared with stock chemicals, solution CF, and the modified Pfeffer's solution. The presence of the filter paper markedly increased the growth in length and production of secondary roots, delayed and reduced the development of abnormalities, and improved the appearance and condition of the roots. While the optimum amount of filter paper was not exactly determined, one-



half or one full sheet of filter paper in 30 cc. of medium appeared to produce the best results. The following experiment (table VII) illustrates the effects of the filter paper. In this case excised corn root tips 1 cm. long were grown individually in 30 cc. of medium in 125 cc. Erlenmeyer flasks for 57 days in the dark at room temperature. The basic medium was solution C containing 2 per cent dextrose. The filter paper was added to the medium before sterilization in the proportion of one-eighth sheet, one-half sheet, or two sheets per flask. While a distinct benefit is evident (fig. 6) from the addition of one-eighth sheet, a much greater effect was secured from the addi-

TABLE VII

EFFECT OF ADDITION OF QUALITATIVE FILTER PAPER TO SOLUTION C ON GROWTH OF EXCISED CORN ROOT TIPS. ORIGINAL LENGTH 1 CM. GROWN 57 DAYS IN 30 CC. OF MEDIUM IN 125 CC. ERLLENMEYER FLASKS IN DARK AT ROOM TEMPERATURE. SHEETS OF FILTER PAPER 9 CM. IN DIAMETER USED

AMOUNT OF FILTER PAPER	No. ROOTS	AV. FINAL LENGTH (CM.)	AV. NO. SECOND- ARY ROOTS	MAXIMUM LENGTH (CM.)	MAXIMUM NO. SEC- ONDARY ROOTS	MINIMUM LENGTH (CM.)	MINIMUM NO. SEC- ONDARY ROOTS
None.....	12	8.8	61	12.5	87	5.1	23
$\frac{1}{8}$ sheet.....	14	26.7	165	68.2	333	7.4	24
$\frac{1}{2}$ sheet.....	12	52.6	223	88.3	320	12.1	64
2 sheets.....	12	44.0	172	88.1	376	14.7	70

tion of one-half sheet. Two sheets appear to be somewhat above the optimum.

The filter paper made solution C somewhat less acid, as may be noted in table VIII, in which the pH after sterilization and the average pH after the growth of the roots are given. A single sheet of the filter paper weighed about 480 mg. and contained approximately 1 mg. of ash.

The effect of the filter paper is due to its water-soluble constituents. This follows because a water extract of the filter paper added to solution C or to the modified Pfeffer's solution improved the growth of the roots about as much as did the filter paper itself.

It is probable that the beneficial effects of the filter paper are due to its ash constituents, since quantitative (ashless) filter paper

(Whatman no. 40, 110 mm. circles) added to solution C in the proportion of one-eighth sheet per flask had no effect and a water extract of quantitative filter paper was ineffective.

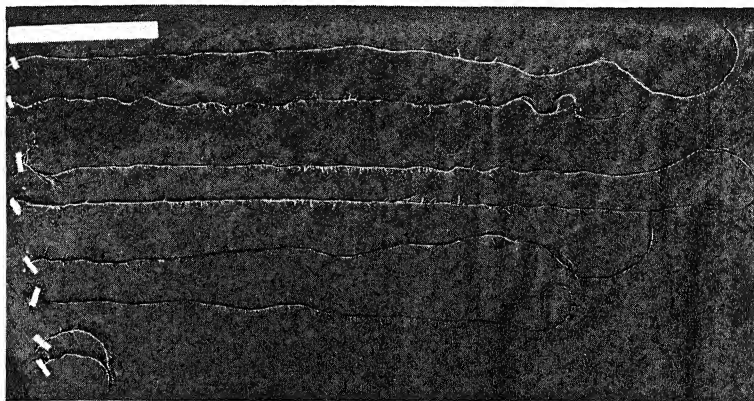


FIG. 6.—Effect of qualitative filter paper on growth of excised corn root tips. From bottom to top: two best roots in solution C; two best in solution C plus  $\frac{1}{8}$  sheet filter paper; two best in solution C plus  $\frac{1}{2}$  sheet; two best in solution C plus 2 sheets. Original length 1 cm.; grown 57 days in 30 cc. of medium at room temperature in the dark.

COTTON AND SOLUBLE STARCH.—The effects of the addition to solution C of cotton and soluble starch were also determined. Absorbent cotton in the proportion of approximately 170 mg. per flask

TABLE VIII

INITIAL AND FINAL HYDROGEN-ION CONCENTRATIONS OF SOLUTIONS  
USED IN EXPERIMENT SUMMARIZED IN TABLE VII

MEDIUM	INITIAL PH	AV. PH AFTER GROWTH OF ROOTS
Solution C.....	4.6	5.2
Solution C + $\frac{1}{8}$ sheet.....	4.75	5.4
Solution C + $\frac{1}{2}$ sheet.....	5.0	5.6
Solution C + 2 sheets.....	5.4	6.1

containing 30 cc. of solution C had little beneficial effect on corn root tips of 1 cm. original length grown at room temperature in the dark. The addition of 1 per cent Merck's soluble starch to solution

C improved the growth and reduced the percentage of abnormal roots. Neither the cotton nor the soluble starch materially changed the pH of solution C.

PRETREATMENTS.—The beneficial effects of the addition to solution C of agar, water extract of agar, qualitative filter paper, or water extracts of qualitative filter paper raised the question as to what a brief treatment with these materials might do. The corn grains were therefore germinated as usual on a water agar and the root tips severed. Some of the severed tips were transferred at once to the culture medium; others were placed for 24 or 48 hours on the

TABLE IX

EFFECT OF PRETREATMENT ON AGAR ON GROWTH OF EXCISED CORN ROOT TIPS.  
ORIGINAL LENGTH 1 CM. GROWN IN 30 CC. OF MEDIUM FOR 41  
DAYS IN DARK AT ROOM TEMPERATURE

MEDIUM	PRETREATMENT	No. ROOTS	AV. FINAL LENGTH (CM.)	AV. NO. SECONDARY ROOTS	MAXIMUM LENGTH (CM.)	MAXIMUM NO. SECONDARY ROOTS	MINIMUM LENGTH (CM.)	MINIMUM NO. SECONDARY ROOTS
Solution C. ....	None	20	8.7	39	17.9	84	4.0	8
Solution C + 0.5% agar. ....	None	11	30.8	148	68.3	340	7.8	21
Solution C. ....	48 hours on agar	13	28.1	133	98.2	470	3.5	20

surface of a semi-solid agar medium or on moist filter paper. After such pretreatment they were transferred to the culture medium.

The pretreatment of 1 cm. root tips on agar for 24 or 48 hours was found to be decidedly beneficial although not so beneficial as the addition of agar or a water extract of agar to the medium. The excised root tips were pretreated in petri dishes on the surface of 0.5 or 0.75 per cent agar made up with solution C with or without dextrose. No experiments were performed in which the root tips were pretreated on a water agar. The results of a typical experiment are given in table IX. In this case excised root tips 1 cm. long were grown individually in 30 cc. of medium in 125 cc. Erlenmeyer flasks for 41 days in the dark at room temperature. The root tips were grown in solution C, in solution C plus 0.5 per cent agar, and in solu-

tion C after pretreatment on agar. The growth in solution C of the pretreated roots was almost as great as that of untreated roots grown in solution C plus 0.5 per cent agar and very much superior to the growth of untreated roots grown in solution C.

Pretreatment of root tips originally 1 cm. in length for 24 or 48 hours on the surface of qualitative filter paper moistened with solution C or solution C without dextrose in petri dishes was also found beneficial, although not so much so as the addition of filter paper to the medium. Pretreatment on filter paper seemed somewhat more effective than pretreatment on agar.

Although pretreatment of roots 1 cm. long was distinctly beneficial, that of roots 2 mm. long seemed to have little effect in the few experiments performed. Pretreatment was not always effective in preventing the development of abnormalities (fig. 3).

To what is the beneficial effect of pretreatments on agar or filter paper due? A number of possibilities suggested themselves and some of them were tested by experiment. The results indicate that the absorption by the roots of something from the agar or the filter paper is responsible for the benefits of the pretreatments.

It was thought that these beneficial effects might be due to the increased oxygen supply during the exposure on the agar or on the filter paper. If this is correct the same beneficial effects should be observed if the excised root tips were exposed in a moist atmosphere in the absence of agar or filter paper. However, such was not the case. The benefit derived from pretreatment on agar or filter paper did not appear to be a matter of increased supply of oxygen. Root tips 1 cm. long exposed for 16 hours in sterile petri dishes containing 2 cc. of redistilled water and transferred to solution C grew no better than those transferred to the solution at once after cutting. Root tips 1 cm. long were placed in sterile petri dishes containing a micro-slide and 4 cc. of solution C without dextrose. They were placed in the dishes in such fashion that the cut basal end rested on the slide in the moist air while the tip was in contact with the liquid. After 24 hours they were transferred to solution C. Here again the exposure to the moist atmosphere was not beneficial.

It was suggested that perhaps the agar or filter paper absorbed some toxic substances formed at the cut base of the excised root.

KEEBLE, NELSON, and SNOW (5) found that washing decapitated roots attached to the grain favored their growth. However, the writers washed excised roots of 1 cm. original length for 45 minutes using 10 cc. of sterile redistilled water for each root and found no benefit when the growth of the washed roots in solution CF was compared with that of roots transferred at once to the solution. Roots washed and then placed on filter paper for 24 hours showed the beneficial effect of the contact with filter paper when grown in solution C.

These experiments on pretreatment in a moist atmosphere or by washing with redistilled water did not support the idea that the beneficial effects of pretreatment on agar or filter paper were due to a greater oxygen supply or to the removal of toxic products from the cut surface. Nevertheless, a further series of experiments was performed in which the excised root tips which had been pretreated on filter paper were freshly cut before being placed in the culture solutions. If the cutting of the root tips in excising them was the cause of the poor growth because of shock or any other reason, and the pretreatment on filter paper in some way neutralized the bad effects, then it seemed that the beneficial effects of pretreatment on filter paper would be eliminated by the fresh cut. However, that was not the case. Excised root tips 1.0 cm. long were pretreated on filter paper for 24 hours. At the end of that time 3 mm. was cut from the base of some of them. All were transferred to solution C and little difference between the resulting growths of the two sets was found.

If the effect of pretreatments on agar or on filter paper is not the result of greater oxygen supply or of the neutralization of the effects of cutting the root, then it would seem that it must be due to the absorption by the root of some substance or substances from the filter paper or the agar. The question at once arises as to why 24 hours on agar after excision should permit more absorption than would occur during the two or three days' contact of root and agar during germination. It is necessary to assume that the materials absorbed from the agar by the root attached to the grain are translocated in part out of the root tip, while in the excised root they accumulate. This would explain why pretreatments are less bene-

ficial for excised root tips 0.2 cm. long than they are for root tips 1.0 cm. long. In the former case there is less opportunity for accumulation because of the smaller amount of tissue.

Although the writers are inclined to explain the beneficial effects of pretreatment on agar or filter paper as due to the absorption of substances from the agar or filter paper, one type of pretreatment found effective cannot easily be explained on that basis. This consisted of placing cut roots 1 cm. long vertically against the inside of a pyrex flask containing solution C in such fashion that the tip touched the liquid but the rest of the root was above the liquid. After 24 hours such roots were washed down into the solution and their growth compared with that of root tips which had been placed in the solution immediately after being severed from the grain. Although the number of roots treated by this method was small the benefits appeared unmistakable, the pretreated roots making between two and three times as much growth as the untreated roots.

OTHER NUTRIENT SOLUTIONS.—WHITE (18) has used a modification of Pfeffer's solution containing cane sugar and dried brewers' yeast for excised tomato root tips and has secured unlimited growth. He found Uspenski's solution also to be a favorable solution. These two solutions were compared by the writers with solution C to which autolyzed yeast had been added. In addition excised corn root tips were grown in the following solution adapted from one used by TRELEASE and TRELEASE (15):

$\text{KH}_2\text{PO}_4$ .....	0.002M
$\text{Ca}(\text{NO}_3)_2$ .....	0.002
$\text{MgSO}_4$ .....	0.001
$\text{NH}_4\text{NO}_3$ .....	0.001
Ferric citrate.....	0.00001

Sufficient  $\text{K}_2\text{HPO}_4$  was added to this solution to bring the hydrogen-ion concentration to pH 5.2.

No difference in total length or production of secondary roots was noted between these solutions. In WHITE's solution comparatively few roots became discolored but the main roots and many of the branches became very thin. In none of these solutions was the growth in length and number of branch roots comparable to that in

solution C which contained agar, agar washings, qualitative filter paper, or filter paper washings.

YEAST.—The beneficial effects of the addition of yeast (either autolyzed yeast or dried brewers' yeast) to the modified Pfeffer's solution originally reported by ROBBINS (9) was confirmed. Similar beneficial effects were noted also when yeast was added to solution C. From the experiments performed, yeast seemed somewhat more effective in solution CF than in solution C, and in solution CF plus agar than in solution C plus agar. The yeast was usually added at a concentration of 400 p.p.m. In one experiment, however, in which autolyzed yeast or dried brewers' yeast was added to solution CF plus 0.5 per cent agar, surprisingly beneficial effects were secured on the growth of 1 mm. root tips by the addition of as little as 1 p.p.m. of the yeast, the brewers' yeast being the more effective. Ten p.p.m. was somewhat less effective, and 50 p.p.m. although still beneficial was not so satisfactory as the 10 p.p.m. Twice as much growth was secured in solution CF plus 0.5 per cent agar containing 1 p.p.m. of dried brewers' yeast as in the medium without the yeast, and one root tip 1 mm. long grew in 71 days to a total length of 61.2 cm. and produced 175 secondary roots. These results are so unexpected that they should be confirmed.

Some experiments were also performed in which yeast ash was added to the modified Pfeffer's solution or to solution C. The yeast ash was dissolved in hydrochloric acid and evaporated to dryness or neutralized with sodium hydroxide. In all cases a benefit from the addition of the ash preparations was found. However, contaminants in the hydrochloric acid used or in the sodium hydroxide may have been responsible in part for the results.

GLUTATHIONE, PANTOTHENIC ACID, VITAMIN B.—Glutathione according to HAMMETT (3) stimulates cell division in roots attached to the seed or grain. The writers found no benefit on the growth of excised roots 1 cm. long from the addition of 0.192, 0.384, 0.96, 1.92, or 3.84 p.p.m. of glutathione to solution C plus 0.5 per cent agar. The roots were grown 55 days in the dark. Glutathione at a concentration of 1.92 p.p.m. did not improve the growth of root tips 2 mm. long in solution C plus 0.5 per cent agar.

WILLIAMS (16) has found pantothenic acid beneficial to the growth

of yeast. Pantothenic acid<sup>4</sup> (potency 70) was somewhat beneficial at a concentration of 50 p.p.m. to the growth of excised root tips 2 mm. long in solution C plus 0.5 per cent agar. In fact the longest root (85.0 cm.) from this original length grew on this medium. At a concentration of 16.6 p.p.m. no effect was noted. Some benefit was found on the growth of root tips originally 1 mm. long in solution CF plus 0.5 per cent agar from the addition of 10 p.p.m. of pantothenic acid (potency 133). No benefits were secured from 1 p.p.m. or from 50 p.p.m. The experiments with pantothenic acid were few in number; and although in some cases benefits were noted from the addition of this material, the results need confirmation.

SCHOPFER (13) has reported that vitamin B greatly improves the growth of *Phycomyces*. A sample of crystalline vitamin B was secured from WILLIAMS<sup>5</sup> and added to solution CF plus 0.6 per cent agar. No benefit was observed in the growth of root tips 1 mm. long from the addition of 0.1, 1, or 2 p.p.m. of vitamin B. The roots were grown for 50 days in the dark.

MAXIMUM GROWTH.—It will be of some interest to record the maximum growth of individual root tips of various original lengths found in the experiments which are discussed in this paper.

The maximum growth of excised corn root tips originally 1 mm. in length was obtained in solution CF plus 0.5 per cent Difco agar and 1 p.p.m. of dried brewers' yeast. In this medium at room temperature in the dark one root grew in 71 days to a total length of 61.2 cm. and produced 175 secondary roots. This is an increase of 0.9 cm. per 24 hours. In solution C plus 0.5 per cent agar the maximum growth was 26.1 cm. with 77 secondary roots.

For root tips 2 mm. long the maximum growth was secured in solution C plus 0.5 per cent agar and 50 p.p.m. pantothenic acid. In this medium at room temperature and in the dark one root grew in 61 days to a total length of 85.0 cm. and produced 244 secondary roots. This is an increase of 1.4 cm. per 24 hours. Long roots were found also in solution C plus 400 p.p.m. of autolyzed yeast (65.3 cm.) and in solution C plus 0.5 per cent agar (53.4 cm.). In solution

<sup>4</sup> Gratitude is expressed to Dr. R. J. WILLIAMS for supplying the material used.

<sup>5</sup> Gratitude is expressed to Dr. R. R. WILLIAMS for the gift of this material.



C after pretreatment on agar the maximum was 9.7 cm. with 45 secondary roots.

The maximum growth of root tips 10 mm. long was secured in solution C after pretreatment on agar. This root at room temperature in the dark grew in 41 days to a length of 98.2 cm. and produced 470 secondary roots, an increase of 2.4 cm. per 24 hours. Long roots were secured also in solution C plus 0.5 per cent agar (92.0 cm. with 448 secondary roots); solution C plus one-eighth, one-half, or two sheets of qualitative filter paper (85.5, 88.3, and 88.1 cm. respectively); solution CF plus 400 p.p.m. of autolyzed yeast (72.2 cm.). The maximum in solution C prepared with the Coleman and Bell dextrose was 25.2 cm. with 168 secondary roots, although a root 64.4 cm. long with 300 secondary roots was grown in solution C prepared with another brand of dextrose of high purity (Pfanstiehl C.P. for injection).

### Discussion

Why do excised corn root tips develop abnormalities and show limited growth in the mineral solutions containing dextrose which were used in these experiments? Conversely, how can excised corn root tips be maintained in a normal and growing condition indefinitely? There is no *a priori* reason why this is not possible of accomplishment. These questions cannot be answered categorically as a result of the experiments reported in this paper, but a discussion of the possibilities is important from the standpoint of further investigation of this problem.

The possible causes for the development of abnormalities and the limited growth in solution C or similar solutions might be enumerated as follows:

1. An injurious condition in the medium because of the presence of something which is toxic, because of an unbalanced condition or some other condition.
2. A nutrient deficiency in the medium which might be lack of sufficient oxygen, a mineral nutrient, or some essential organic substance or substances which the root does not synthesize.
3. The condition of the excised root at the time of transfer; a condition resulting from its genetic constitution, its previous history, or

from some phase of the treatment in preparing or excising the root tip.

4. Some environmental factor not included in the medium (for example, temperature of incubation).

If the results are due to an injurious condition in the medium, the distilled water would not seem to be at fault since little difference was found in the effects of different kinds of distilled or redistilled water. It would not appear to be due to the hydrogen-ion concentration, which was normally near pH 4.5, since making the solutions used less acid by the addition of KOH or  $K_2HPO_4$  did not improve conditions. It would not appear to be due to any contaminant in the mineral salts used since purified salts gave no better results than the stock chemicals. It is not due to changes in the dextrose caused by heating in sterilization since dextrose filtered sterile was no better than dextrose sterilized by heat. It is possible that some contaminant in the dextrose is responsible. This is not probable since samples of dextrose of high purity were used and further, cane sugar of highest purity was not superior to dextrose when substituted for the latter. It may be due to a lack of balance in the mineral solution although some variation in the proportions of salts was without marked benefit. In fact we are not inclined at this time to ascribe our results to any toxic condition in solution C, in the modified Pfeffer's solution or in similar solutions, although it is recognized that limited growth and abnormalities can be produced by toxic substances.

It does not seem likely that the limited growth and abnormalities result from the treatment of grains or roots before placing the root tips in the culture flasks. This treatment includes the sterilization of the grains, their germination, the excision of root tips and their transfer to the culture flasks. That sterilization of the grains is not a factor is indicated by the results reported by ROBBINS and MANEVAL (10) with roots secured from unsterilized grains, and by the fact that roots of grains grown in water culture show no differences whether sterilized or not sterilized with calcium hypochlorite. The temperature of germination was 26° C., and the medium (water agar) upon which the grains germinated was favorable for growth, as demonstrated by the appearance of the roots and by the cultures

to which agar or agar washing had been added. The conditions of germination, therefore, do not appear to be involved. Neither does the cutting of the root seem important. No difference was found in the effects on roots of the lengths used in these experiments of a thin or a thick knife blade used in excising the roots. Washing the roots after excision or the removal of a fresh piece of the root after pre-treatment did not affect the resulting growth.

The environmental conditions other than the culture medium itself do not seem to be involved. The temperature at which the cultures were incubated was not controlled and varied between 19° and 30° C. with an average of 25° C. The higher temperatures are unfavorable but most of the culture work was carried on during the fall, winter, and spring when the temperatures were from 20° to 22° C. Light under some circumstances is a favorable factor (11). It does not, however, overcome the effects of solution C or similar solutions.

The osmotic pressure of our solutions is about 0.11 mol., less than half the molecular concentration which causes plasmolysis of corn root hairs (12).

The possibility that the limited growth in solution C or similar solutions is the result of a deficiency in the medium seems most probable. If such is the case then it is not due to a deficiency of water since the roots are grown in liquid cultures, nor to a lack of carbohydrate since there is a large amount of dextrose in the solution at the termination of the experiments. A deficiency of oxygen is of some significance. This is shown by the beneficial effects of exposing root tips to the air by tilting the flask or by pulling the root tips temporarily out of the liquid medium. Lack of oxygen is not alone responsible for the results in solution C, however, since the addition to the liquid medium of agar washings, qualitative filter paper, qualitative filter paper washings, soluble starch, and the use of certain brands of dextrose produce more improvement than any of the methods used to increase the oxygen supply to the roots. It is possible, of course, that the additions referred to facilitate the respiratory activity of the root even though the oxygen supply is actually not increased. If the limited growth and development of abnormalities in solution C or similar solutions are due to a de-

ficiency in the medium it is a deficiency which is supplied wholly or in part by peptone, yeast, agar, filter paper, soluble starch, and some contaminant in certain brands of dextrose. It is difficult although not impossible to conceive of these various materials supplying an organic deficiency; a mineral deficiency seems more likely. If such is the case it is not a deficiency of phosphorus, sulphur, magnesium, potassium, calcium, copper, or thallium. It is probably not a deficiency of iron. Some improvement was observed by the addition of a mixture of salts of manganese, boron, and zinc but not as much as by the addition of agar or filter paper. Further experiments should be performed in which it is definitely determined whether the beneficial effects of peptone, yeast, agar, filter paper, and soluble starch are due to organic or to inorganic constituents; and the effect of various rarer elements should be tested.

It is entirely possible that more than one factor may be found important in making a synthetic solution such as solution C adequate for the growth of excised corn root tips. Not only may mineral elements other than those contained therein be necessary, but organic nitrogen (such as amino acids) or other organic substances may be significant.

Are any of the various media used in these experiments entirely adequate for the growth of excised corn roots? It is probable that one or more of them is, although the solution found by WHITE (18) as satisfactory for unlimited growth of tomato roots does not appear from our results satisfactory for excised corn root tips. However, in these experiments we have not attempted to investigate directly the problem of unlimited growth of excised corn roots. It may be pointed out in this connection that not only do the plants used by WHITE and ourselves differ but that we have been concerned with the meristem of the main root while WHITE has used meristems of secondary roots as well. The use of the growing points of secondary roots greatly augments the possibilities of the continued growth of tissue from a single isolated tip by multiplying many times the number of cultures containing meristem, and thus permitting the continuance of cultures from a root in which the meristem of the primary root tip has ceased to grow. In addition, the use of subcultures serves a selective process by eliminating at the start those

roots which for some genetic or other reason do not adapt themselves to growth in the medium used.

The possible advantage of the cultivation of excised root tips or other tissues of higher plants for the investigation of the nutritional requirements of higher plants and their development is obvious. An excised root tip in contrast to a seed is far more limited in its stored supply of mineral nutrients and organic constituents, and furthermore is not supplied during its development by the products formed by the photosynthetic activity of the leaves. By proper refinements of technique it seems entirely feasible to contribute by these methods to the problems involved in the mineral nutrition of higher plants, the synthesis of proteins, and the possible function of accessory growth substances.

### Summary

1. Excised corn root tips grown in a liquid medium (solution C) composed of calcium nitrate, magnesium sulphate, potassium dihydrogen phosphate, ferric chloride, and dextrose showed limited growth and sometimes became abnormal. Similar results were secured in a modified Pfeffer's solution containing dextrose.

2. The amount of growth in solution C varied with the brand of dextrose used, even when the dextrose was of high purity.

3. The substitution of levulose, cane sugar, xylose, or maltose for dextrose in solution C was of no benefit; in fact, the growth with xylose and maltose was very poor.

4. Growth was improved by the addition to solution C of Difco agar, water extracts of agar, qualitative filter paper, water extracts of qualitative filter paper, soluble starch, autolyzed or dried brewers' yeast, or a mixture of salts of manganese, boron, and zinc.

5. The best results were secured by the addition of qualitative filter paper in the proportion of one half sheet, 9 cm. in diameter, to 30 cc. of medium.

6. Exposure of excised root tips originally 1 cm. long for 24 or 48 hours on the surface of a semi-solid agar medium or on moist qualitative filter paper markedly favored their later growth in solution C.

7. Exposure of excised root tips in a moist atmosphere or washing them in redistilled water before cultivation in solution C was not beneficial.

8. Recutting excised roots after pretreatment on filter paper did not affect their later growth in solution C.

9. Increased aeration of the root tips in the liquid medium increased the growth and delayed the occurrence of abnormalities. The results were not so favorable as those secured by the addition of agar or of filter paper to the medium.

10. Reducing the acidity of the solution, increasing its salt concentration, substituting ferric tartrate or ferric citrate for ferric chloride, adding quantitative filter paper, absorbent cotton, copper sulphate, thallium nitrate, glutathione, or vitamin B to the medium were not beneficial.

11. The addition of pantothenic acid was in some cases beneficial.

12. Pfeffer's solution, Uspenski's solution, and the solution used by WHITE for the cultivation of excised tomato roots were no more effective than solution C.

13. The maximum growth of individual excised corn root tips 1 mm. long was 61.2 cm., or 0.9 cm. per 24 hours; of root tips 2 mm. long was 85.0 cm., or 1.4 cm. per 24 hours; of root tips 10 mm. long was 98.2 cm., or 2.4 cm. per 24 hours.

14. It is thought that the poor growth in solution C and similar solutions is the result of a deficiency in the solution, a part of which at least is inorganic.

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# EFFECT OF ALTERNATE MOISTENING AND DRYING ON GERMINATION OF SEEDS OF WESTERN RANGE PLANTS

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 475<sup>1</sup>

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(WITH NINE FIGURES)

## Introduction

Although considerable study has been given to the germination behavior of various seeds, little is known regarding the effect that alternation of moistening and drying has upon their germination. In times of drought the seeds dry out and lie in that condition for an indefinite period. In preliminary tests conducted with the seeds of a large number of range plants from the Wasatch Plateau in central Utah, some of the seeds gave high percentages of germination under ordinary germinating conditions with continuous moisture at room temperature; others gave little or no germination under such conditions.

## REVIEW OF LITERATURE

The literature includes few references concerning the seeds of range plants or the effect of drying after the seeds have absorbed water. SAMPSON (29, 30, 31, 32) reports the percentage of viability in germination tests of the seeds of a number of range plants<sup>2</sup> from

<sup>1</sup> In cooperation with the Intermountain Forest and Range Experiment Station of the United States Department of Agriculture, Forest Service.

<sup>2</sup> Owing to changes in nomenclature or in the check identification of certain species from the Manti National Forest by the Bureau of Plant Industry, ten of the species studied by SAMPSON are discussed in this report under other names:

*Agropyron pauciflorum* (Schwein.) Hitchc. (*A. tenerum* Vasey) not *A. violaceum* (Hornem.) Lange

*Bromus anomalus* Rupr. (*B. porteri* [Coul.] Nash.)

*Bromus polyanthus* Scribn. (*B. marginatus* Nees)

*Melica bulbosa* Geyer (*M. bella* Piper)

*Poa secunda* Presl. (*P. sandbergii* Vasey)

*Stipa columbiana* Macoun (*S. minor* Scribn.)

[Footnote continued on p. 244]



the Manti National Forest in central Utah, and the Wallowa National Forest in northeastern Oregon. Fourteen of the species which he studied (seven of which were from the Manti National Forest) are included in this investigation. In the tests conducted by SAMPSON most of the seeds gave low percentages of germination, but unless the conditions requisite for germination of each particular species are supplied, the results do not show the true viability of the seeds. The lack of adequate data regarding the conditions used in SAMPSON's tests makes a comparison of results impossible.

Several investigators have studied the effect of reduced moisture content on germination, using fresh seeds for desiccation. EWART (10) concluded that the moisture content could not be reduced below 2 or 3 per cent without seriously affecting the vitality of the seeds. Others (9, 14, 25, 28, 33, 35) found that the water content could be reduced below this point, in some instances to a mere trace of moisture, with little or no effect on subsequent germination. ATTERBERG (1) observed that drying immature barley seeds at 37° C. increased the percentage of germination over seeds dried at lower temperatures, but drying soaked seeds of high germinative capacity at 48° to 50° C. or higher, reduced the percentage of germination.

From experiments with *Brassica alba*, KIDD (20) and KIDD and WEST (21) discovered that drying seeds during an early stage of development previous to germination accelerated germination when the seeds were remoistened, but drying the seeds after cell division had advanced resulted in injury. They found also that secondary dormancy was broken by complete redrying, or by removal of the seed coat, followed by remoistening. They interpret such germination as the result of mechanical stimulus on the embryo and not as changes within the seed coat, although removing the coats gave 100 per cent germination within a day after moisture was supplied. The effect of desiccation on the seed coats of certain water plants (4, 23) has long been known to be beneficial to germination.

The seed coat, according to CROCKER (3, 4, 5, 6), is most frequent-

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*Artemisia incompta* Nutt. not *A. discolor* Dougl.

*Lepidium densiflorum* Schrad. (*L. ramosissimum* A. Nels.)

*Penstemon rydbergii* A. Nels. not *P. procerus* Dougl.

*Pseudocymopterus montanus* (A. Gray) Coult. and Rose (*P. tidestromii* Coult. and Rose).

ly the cause of delayed germination. It may prevent the absorption of water (11, 13, 15, 16, 26) or oxygen (2, 3, 34), or may mechanically restrain the embryo from developing (6, 7). The seeds of *Amaranthus retroflexus* (6), *Avena fatua* (2, 36), and some of the grains (1, 22) have been found to germinate better after a period of dry storage. CROCKER interprets the changes in *Amaranthus retroflexus* as due to hysteretic changes in the colloids of the seed coat, which lower the elasticity and the breaking strength of the coat (6). Changes in other seeds might harden the seed coat or affect the permeability to oxygen or water, as observed by DUVEL (9). Not only the seeds of different species, but the seeds of a given species may also vary in their oxygen requirement (3, 34). CROCKER (3) found that the rate of diffusion of oxygen through the seed coat of *Xanthium* was increased when the coat was allowed to become somewhat dry.

MUNERATI and ZAPPAROLI (24) investigated the effect of alternately moistening and drying the seeds of several noxious weeds. For 17 months they subjected the seeds to short (15 days) and long (3 months) periods of drying following a 15-day period of moistening. In four species germination was increased materially by both short and long periods of drying, while with two species there was only a slight increase. Drying decreased the germination of one species and had no effect upon another.

#### PURPOSE OF INVESTIGATION

The purpose of this investigation was to seek information concerning the following questions: What effect would an alternation of moistening and drying for short periods have upon the seeds of the different species of range plants? Would it hasten or delay the germination of seeds exposed to such conditions? Would it affect the percentage of germination? Might it have a beneficial effect upon the germination of seeds which gave little or no germination with continuous moisture at room temperature, or would the seeds be injured? Would seeds dried after being in moist conditions for some time without germinating, still retain their viability; that is, would seeds in which the processes of after-ripening and embryo development were interrupted by drying, germinate when again supplied with moisture?

## Materials and methods

## SPECIES STUDIED

Many of the "seeds" studied are fruits rather than seeds, but for the sake of convenience, the terms seed and seed coat are used regardless of the origin of the enveloping structures. Seeds of the following 42 species<sup>3</sup> were chosen to study the effect of alternate moistening and drying for short periods. For the long period of drying only 25 of the 42 species were used.

GRASSES.—*Agropyron pauciflorum* (Schwein.) Hitchc. (*A. tenerum* Vasey), *Bromus anomalus* Rupr. (*B. porteri* [Coul.] Nash), *B. polyanthus* Scribn., *Hordeum nodosum* L., *Melica bulbosa* Geyer, *Poa interior* Rydb., *P. secunda* Presl. (*P. sandbergii* Vasey), *Stipa columbiana* Macoun, and *S. lettermani* Vasey.

HERBS OTHER THAN GRASSES.—*Achillea lanulosa* Nutt., *Androsace diffusa* Small., *Artemisia incompta* Nutt., *Castilleja sulphurea* Rydb., *Chenopodium album* L., *Collomia linearis* Nutt., *Delphinium barbeyi* Huth., *Erythronium parviflorum* (S. Wats.) Goodding, *Frasera speciosa* Dougl., *Geranium viscosissimum* Fisch. & Mey., *Gilia pulchella* Dougl., *Lepidium densiflorum* Schrad., *Lupinus parviflorus* Nutt., *Moldavica parviflora* (Nutt.) Britton, *Orthocarpus tolmiei* Hook & Arn., *Pentstemon rydbergii* A. Nels., *P. subglaber* Rydb., *Plantago tweedyi* A. Gray, *Polemonium foliosissimum* A. Gray, *Polygonum douglasii* Greene, *Pseudocymopterus montanus* (A. Gray) Coul. & Rose, *Rudbeckia occidentalis* Nutt., *Rumex mexicanus* Meisn., *Sophia hartwegiana* (Fourn.) Greene, *Vicia americana* Muhl., and *Viola linguaefolia* Nutt.

WOODY SPECIES.—*Acer grandidentatum* Nutt., *Cercocarpus montanus* Raf., *Chrysothamnus lanceolatus* Nutt., *Ribes inebrians* Lindl., *R. montigenum* McClatchie, *Sambucus microbotrys* Rydb., and *Symphoricarpos oreophilus* A. Gray.

The form of *Agropyron pauciflorum* studied is the wheatgrass heretofore incorrectly known as *A. violaceum*. *Bromus polyanthus* is considered by HITCHCOCK (17) to be only a form of *B. carinatus*, but

<sup>3</sup> Identification of all species has been checked with specimens in the herbarium of the Great Basin Branch Station, which have been identified by the Bureau of Plant Industry; or when there has been any question of doubt, the plants themselves have been checked by the Bureau of Plant Industry.

in accordance with the older usage, it is here treated as a species. *Achillea lanulosa* includes the subspecies *A. lanulosa alpicola* Rydb.

#### COLLECTION AND STORAGE

The seeds were collected from plants in their natural habitat after they had ripened but prior to dissemination, except a few seeds of *Collomia*, *Polygonum*, and *Viola* which were taken from the ground soon after dissemination. The collections were made between August 16 and September 17, 1928, in the region of the Great Basin Branch of the Intermountain Forest and Range Experiment Station on the Manti National Forest in central Utah. The seeds of each species were collected from definite areas. In the case of *Poa secunda*, collections were made from two areas. These were kept separate and are designated as *P. secunda* no. 1 and *P. secunda* no. 2. The seeds from dry fruits were placed directly in paper containers. Those from fleshy fruits were washed free from pulp and air-dried before they were placed in paper containers. All seeds were later placed in bottles with cork stoppers and stored at room temperature.

#### GERMINATION TESTS

The germination tests were made in the laboratories of the University of Chicago during a period extending from December 1928 to February 1930.

In preparation for these tests the seeds were disinfected in a 0.25 per cent solution of uspulun for 30 minutes and then rinsed in sterile water twice for 15 minutes each. They were then placed on sterile, moist cellucotton in petri dishes. Usually 50 seeds of a given species were used in each test, but for *Erythronium*, *Frasera*, *Geranium*, *Lupinus*, *Polygonum*, *Vicia*, *Acer*, and *Cercocarpus* 20 or 25 seeds were often used. As soon as the seeds germinated the seedlings were counted and discarded. Seeds were considered to have germinated when the hypocotyl protruded through the seed coat to an extent of 5 mm. or more.

To study the effect of short periods of alternate moistening and drying on the germination of the seeds included in this investigation, three sets of seeds were used, all of which were kept at room temperature. During the period of the experiment the temperature of the

room varied between 22° and 29° C. The temperatures used for drying are considerably lower than those used by ATTERBERG in his experiments with barley.

In one set of seeds (*A*), the substrate was kept moist continuously during the period of the test. In the second set (*B*) the substrate was kept moist for 5-7 days, then allowed to dry out by opening the petri dishes to permit evaporation. The dishes were left open until the cellucotton appeared thoroughly dry, then the substrate was remoistened and the dishes closed for three days, after which they were opened and the contents again allowed to dry. This process of alternate moistening and drying was repeated until the test had run for 51 to 53 days. Each period of drying required two or three days, depending upon the humidity of the atmosphere at the time. As an aid in preventing mold and bacterial growth, the petri dishes were kept in a case with cloth-covered sides which permitted the circulation of air but kept out dust and other foreign material.

The third set of seeds (*C*) was also subjected to alternate moistening and drying, but the period of moistening preceding the period of drying was shorter than that used in test *B*. Drying also took place more slowly. This set of seeds was not placed in conditions for germination until after the close of test *B*. The seeds were prepared as for *A* and *B* and placed on moist cellucotton for one day, after which the petri dishes were opened to dry. A period of high humidity prevailed at the time; consequently evaporation took place slowly and 12 days were required to dry out the substrate and seeds. They were then remoistened for one day and again allowed to dry out, which required four days. After a further period of three days under moist conditions the test was discontinued.

At the end of the period of alternate moistening and drying in both tests *A* and *B*, many of the seeds which had not germinated still looked healthy. With the exception of two species, *Lepidium* and *Rumex*, the seeds which had not germinated in test *B* were placed immediately in other conditions for germination to determine whether they had retained their viability. The seeds of *Lepidium* and *Rumex* were left in alternating moisture conditions for an additional 20 days. The seeds of the remaining species from test *B* were

remoistened, placed at 0° to 3° C. for eight days, then changed to room temperature of 22° to 29° C. for 12 days. At the end of the 12-day period the seeds of all but the following ten species were discarded. The seeds of *Delphinium*, *Pentstemon subglaber*, and *Ribes inebrians* were dried and kept for a later test. The seeds of seven species, *Collomia*, *Erythronium*, *Frasera*, *Gilia*, *Orthocarpus*, *Polemonium*, and *Polygonum*, were left for a further period of 120 days in a Minnesota germinator at 22° to 29° C. Following this long period at warm temperatures *Collomia* and *Frasera* were placed directly at alternating temperatures. The seeds of the other five species, *Erythronium*, *Gilia*, *Orthocarpus*, *Polemonium*, and *Polygonum*, were placed at cold temperatures of 0° to 3° C. for 34 days, then placed at alternating temperatures.

From test C the seeds of only five species were kept for further study. *Agropyron*, *Hordeum*, *Rumex*, *Viola*, and *Symphoricarpos* seeds were dried for 105 days to be used with other seeds in the following test.

To determine the effect of a long period of drying upon seeds which had been in moist conditions for some time, seeds which had failed to germinate under various conditions extending from 21 to 204 days were air-dried and kept at room temperature in paper containers for 105 days. Forty-eight samples representing 25 species were used. Three of the 48 samples were from the set of seeds subjected to alternating moisture conditions for 51 to 53 days (test B) and five samples were from those seeds which had been subjected to alternating moisture conditions for 21 days (test C), as previously noted. The remaining 40 samples were taken from seven other germination tests in all of which the seeds were kept moist continuously, but in which different temperature conditions had been used.

After drying for 105 days, the seeds were disinfected as for other tests and placed in petri dishes on sterile moist cellucotton. The seed coats of one set of *Vicia* seeds were broken by filing before they were placed in moist conditions. The seeds of each species were then placed under temperature conditions which were known or considered to be favorable for germination. The following were the temperature conditions used:

TABLE I  
EFFECT OF ALTERNATE MOISTENING AND DRYING OF  
SEEDS ON GERMINATION

SPECIES	PERCENTAGE GERMINATION			
	WITH CON- TINUOUS MOISTURE	UNDER ALTERNATING MOISTURE CONDITIONS		MAXIMUM GERMINATION OBTAINED IN OTHER TESTS
		RAPID DRYING	SLOW DRYING	
		TEST B	TEST C	
Grasses				
Agropyron pauciflorum.....	6	2	4	86
Bromus anomalus.....	94	80	94	100
B. polyanthus.....	98	100	98	100
Hordeum nodosum.....	0	0	0	100
Melica bulbosa.....	0	0	0	94
Poa interior.....	96	98	80	100
P. secunda no. 1.....	64	68	40	86
P. secunda no. 2.....	16	20	2	58
Stipa columbiana.....	38	34	32	84
S. lettermani.....	10	26	16	98
Herbs other than grasses				
Achillea lanulosa.....	82	98	36	100
Androsace diffusa.....	52	62	10	38
Artemisia incompta.....	58	.....	76	86
Castilleja sulphurea.....	0	0	2	10
Chenopodium album.....	12	10	2	22
Collomia linearis.....	0	0	0	62
Delphinium barbeyi.....	0	0	0	2
Erythronium parviflorum.....	0	0	0	70
Fraseria speciosa.....	0	0	0	45
Geranium viscosissimum.....	100	95	85	100
Gilia pulchella.....	0	0	0	32
Lepidium densiflorum.....	48	90	48	100
Lupinus parviflorus.....	95	70	75	100
Moldavica parviflora.....	2	2	0	4
Orthocarpus tolmei.....	0	0	0	42
Pentstemon rydbergii.....	4	16	0	30
P. subglaber.....	0	2	0	22
Plantago tweedyi.....	80	84	98	100
Polemonium foliosissimum.....	0	0	0	30
Polygonum douglasii.....	0	0	0	5
Pseudocymopterus montanus.....	86	72	46	88
Rudbeckia occidentalis.....	60	44	90	98
Rumex mexicanus.....	22	52	0	100
Sophia hartwegiana.....	2	2	2	6
Vicia americana.....	0	5	5	100
Viola linguaeifolia.....	0	0	0	2
Woody species				
Acer grandidentatum.....	0	0	0	53
Cercocarpus montanus.....	5	10	10	65

TABLE I—*Continued*

SPECIES	PERCENTAGE GERMINATION			
	WITH CONTINUOUS MOISTURE	UNDER ALTERNATING MOISTURE CONDITIONS		MAXIMUM GERMINATION OBTAINED IN OTHER TESTS
		RAPID DRYING	SLOW DRYING	
		TEST B	TEST C	
Chrysothamnus lanceolatus . . . . .	86	64	48	80
Ribes inebrians . . . . .	0	0	0	98
R. montigenum . . . . .	0	0	0	17
Sambucus microbotrys . . . . .	0	0	0	6
Symphoricarpos oreophilus . . . . .	0	0	0	32

1. Cold, 0° to 3° C. for the period of the test.
2. Alternating temperatures, 0° to 3° C. and 17° to 22° C. for approximately 12 hours each during the period of the test.
3. Cold, 0° to 3° C. for 8 days, then 22° to 27° C. until the end of the test.
4. Cold, as preceding for 8 days, then alternating temperatures as above until the end of the test.

The seeds were left in these conditions for 76 to 91 days.

### Results

Tables I to IV give the results of the tests on the effect of short periods of alternate moistening and drying on the germination of the seeds included in this investigation. Table I gives for each species the various percentages of germination. The last column gives the maximum percentage of germination obtained in various other tests made from the same lots of seeds during a period extending from December 1928 to February 1932. The complete results of these tests will be published in a subsequent paper. While the results in the last column do not give a true index of viability for all of the species studied, they do provide a basis for comparison with the results obtained from the seeds under conditions of alternating moisture.

The seeds of the following 16 species gave no germination at room



temperatures, either with continuous moisture or with alternate moistening and drying. *Acer grandidentatum*, *Collomia linearis*, *Delphinium barbeyi*, *Erythronium parviflorum*, *Frasera speciosa*, *Gilia*

TABLE II  
COMPARISON OF PERCENTAGE AND TIME OF GERMINATION OF  
SEEDS UNDER THREE CONDITIONS OF MOISTURE

SPECIES	GERMINATION					
	CONTINUOUS MOISTURE		ALTERNATING MOISTURE CONDITIONS			
			RAPID DRYING		SLOW DRYING	
	DAYS REQUIRED	PERCENT- AGE	DAYS REQUIRED	PERCENT- AGE	DAYS REQUIRED	PERCENT- AGE
Grasses						
Bromus anomalus.....	5-25	94	3-34	80	4-14	94
B. polyanthus.....	3-16	98	3-25	100	4-12	98
Poa interior.....	3-13	96	3- 8	98	4-21	80
P. secunda no. 1.....	3-37	64	3-16	68	4-10	40
P. secunda no. 2.....	5-30	16	5-16	20	14	2
Stipa columbiana.....	3-39	38	3-25	34	4-12	32
S. lettermani.....	22-37	10	11-34	26	7-18	16
Herbs other than grasses						
Achillea.....	2-52	82	4-28	98	7-21	36
Androsace.....	4-26	52	9-52	62	7	10
Artemisia.....	4-46	58	.....	.....	4-14	76
Chenopodium.....	2- 9	12	2-12	10	21	2
Geranium.....	3-18	100	5-20	95	4-21	85
Lepidium.....	3-51	48	3-53	90	4-21	48
Lupinus.....	5-53	95	10-48	70	4-21	75
Pentstemon rydbergii...	9-23	4	13-48	16	.....	0
Plantago.....	3- 6	80	7-13	84	4-18	98
Pseudocymopterus.....	9-28	86	13-29	72	12-21	46
Rudbeckia.....	3- 9	60	7-10	44	4-13	90
Rumex.....	23-25	22	24-53	52	.....	0
Woody species						
Chrysothamnus.....	6-49	86	10-44	64	4-21	48

*pulchella*, *Hordeum nodosum*, *Melica bulbosa*, *Orthocarpus tolmiei*, *Polemonium foliosissimum*, *Polygonum douglasii*, *Ribes inebrians*, *R. monigenum*, *Sambucus microbotrys*, *Symphoricarpos oreophilus*, *Viola linguaefolia*.

The following seven species gave from 2 to 10 per cent only: *Agropyron pauciflorum*, *Castilleja sulphurea*, *Cercocarpus montanus*,

*Moldavica parviflora*, *Pentstemon subglaber*, *Sophia hartwegiana*, *Vicia americana*.

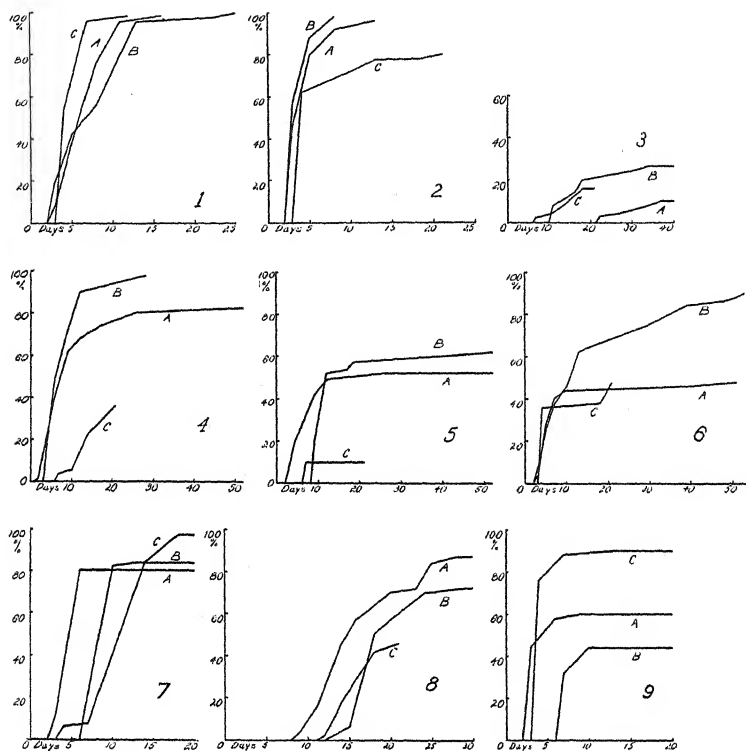
The time required for the germination of the seeds in tests *A*, *B*, and *C*, with the percentage of germination obtained for each of the 19 species which gave over 10 per cent germination at room temperatures, is shown in table II.

TABLE III  
EFFECT OF ALTERNATE MOISTENING AND DRYING  
ON PERCENTAGE OF GERMINATION

SPECIES	PERCENTAGE GERMINATION			
	INCREASE WITH RAPID DRYING	INCREASE WITH SLOW DRYING	DECREASE WITH RAPID DRYING	DECREASE WITH SLOW DRYING
<i>Bromus anomalus</i> .....		2	14	
<i>B. polyanthus</i> .....	2	0		0
<i>Poa interior</i> .....	2			16
<i>P. secunda</i> no. 1.....	4			14
<i>P. secunda</i> no. 2.....	4			10
<i>Stipa columbiana</i> .....			4	4
<i>S. lettermani</i> .....	16	16		
<i>Achillea lanulosa</i> .....	16			38
<i>Androsace diffusa</i> .....	10			40
<i>Artemisia incompa</i> .....		26		
<i>Chenopodium album</i> .....			2	10
<i>Geranium viscosissimum</i> .....			5	15
<i>Lepidium densiflorum</i> .....	42	4		
<i>Lupinus parviflorus</i> .....		5	25	
<i>Pentstemon rydbergii</i> .....	12			2
<i>Plantago tweedyi</i> .....	4	18		
<i>Pseudocymopterus montanus</i> .....			14	24
<i>Rudbeckia occidentalis</i> .....		30	16	
<i>Rumex mexicanus</i> .....	30			22
<i>Chrysothamnus lanceolatus</i> .....			22	2

The influence of alternate moistening and drying as shown by increase or decrease in percentage of germination is given in table III. Since the test in which the seeds were dried slowly (*C*) extended over a period of 21 days only, and tests *A* and *B* extended over 51 to 53 days, the final results of test *C* are not comparable with the other two except in six species, *Poa interior*, *P. secunda* no. 2, *Chenopodium*, *Geranium*, *Plantago*, and *Rudbeckia*, in which germination occurred within 21 days. However, the effect of alternating moisture

conditions on the percentage and the rate of germination during the period of test C and the early period of tests A and B may be compared. The increase or decrease in percentage of germination of the



FIGS. 1-9.—Graphs showing rate and percentage of seed germination: A, given continuous moisture; B, subjected to alternating moisture conditions in which drying was rapid; C, subjected to alternating moisture conditions with slow drying. Fig. 1, *Bromus polyanthus*; fig. 2, *Poa interior*; fig. 3, *Stipa lettermani*; fig. 4, *Achillea lanulosa*; fig. 5, *Androsace diffusa*; fig. 6, *Lepidium densiflorum*; fig. 7, *Plantago tweedyi*; fig. 8, *Pseudocymopterus montanus*; fig. 9, *Rudbeckia occidentalis*.

seeds from test C is based, therefore, on the germination of seeds which were kept moist continuously (test A) for a period of 21 days, and not on the final percentage of germination. Decreased germination was accompanied by a delay and increased germination by hastening, except with *Poa secunda* no. 1, *Androsace*, *Plantago*, *Rudbeckia*, and *Rumex*, in which a delay at first was followed later

by increased germination. In *Rudbeckia* and *Rumex* the retardation was slight. Differences of 5 per cent or less in germination results are not considered of importance. Figures 1 to 9 show the percentage and rate of germination in each of nine species representative of the different ways in which the seeds were affected.

TABLE IV  
GERMINATION OF SEEDS FROM TEST B AFTER PERIOD  
OF ALTERNATE MOISTENING AND DRYING

SPECIES	PERCENTAGE GERMINATION		
	DURING PERIOD OF ALTERNATE MOISTENING AND DRYING	AFTER PERIOD OF ALTERNATE MOISTENING AND DRYING	TOTAL
Grasses			
Agropyron pauciflorum.....	2	20	22
Hordeum nodosum.....	0	74	74
Poa secunda no. 1.....	68	2	70
P. secunda no. 2.....	20	6	26
Herbs other than grasses			
Achillea lanulosa.....	98	2	100
Collomia linearis.....	0	62	62
Frasera speciosa.....	0	5	5
Gilia pulchella.....	0	8	8
Lepidium densiflorum.....	90	2	92
Lupinus parviflorus.....	70	5	75
Pentstemon rydbergii.....	16	6	22
P. subglaber.....	2	12	14
Plantago tweedyi.....	84	2	86
Polemonium foliosissimum.....	0	20	20
Polygonum douglasii.....	0	5	5
Rumex mexicanus.....	52	2	54
Vicia americana.....	5	30	35
Woody species			
Chrysothamnus lanceolatus.....	64	2	66
Ribes inebrians.....	0	2	2

GERMINATION OF SEEDS AFTER ALTERNATE  
MOISTENING AND DRYING

Some of the seeds which failed to germinate when subjected to alternating moisture conditions with rapid drying (test B) germinated when placed in other conditions following the period of alternate moistening and drying (table IV).

TABLE V  
GERMINATION OF SEEDS SUBJECTED TO LONG PERIOD OF DRYING

SPECIES	No. SEEDS USED	FIRST GERMINATING CONDITIONS	PER- CENT- AGE GERMI- NATION	No. SEEDS DRIED	GERMINATING CONDITIONS AFTER DRYING	PER- CENT- AGE DRIED SEEDS GERMI- NATING
Agropyron.....	50	Alternating moisture 21 days	4	48	Cold, then warm	41
Hordeum.....	50	23° to 25° 35 "	4	42	Alt. temp.	97
Hordeum.....	50	Alternating moisture 21 "	0	49	Alt. temp.	96
Melica.....	50	-1.5° to -2.5° 10 "	0	49	Cold	94
Melica.....	50	23° " 25° 25 "	0	49	Cold	84
Melica.....	50	19° " 24° 35 "	0	49	Cold	92
Androsace.....	50	22° " 29° 74 "	0	46	Alt. temp.	20
		3° " 7° 96 "				
		17° " 21° 51 "				
Castilleja.....	50	3° " 7° 96 "	2	45	Cold, then alt. temp.	4
		17° " 21° 51 "				
Castilleja.....	50	22° " 29° 74 "	0	36	Cold, then alt. temp.	0
Chenopodium....	50	22° " 29° 74 "	12	41	Cold, then alt. temp.	2
Chenopodium....	50	19° " 24° 35 "	8	42	Cold, then alt. temp.	7
Chenopodium....	50	3° " 7° 96 "	18	41	Cold, then warm	0
		17° " 21° 51 "				
Collomia.....	50	22° " 29° 74 "	0	41	Cold, then alt. temp.	7
Collomia.....	50	3° " 7° 96 "	40	29	Cold, then alt. temp.	13
		17° " 21° 51 "				
Delphinium.....	50	3° " 7° 96 "	2	48	Cold, then warm	0
		17° " 21° 51 "				
Delphinium.....	50	Alternating moisture 53 "	0	27	Cold, then alt. temp.	0
Erythronium....	20	22° to 29° 74 "	0	19	Cold, then alt. temp.	0
Frasera.....	20	22° " 29° 74 "	0	16	Cold, then alt. temp.	25
Gilia.....	50	22° " 29° 74 "	0	23	Cold, then alt. temp.	9
Gilia.....	25	3° " 7° 96 "	0	24	Cold, then warm	12
		17° " 21° 51 "				
Lupinus.....	20	3° " 7° 96 "	90	2	Cold, then warm	50
		17° " 21° 51 "				
Moldavica.....	50	22° " 29° 74 "	2	48	Alt. temp.	0
Moldavica.....	50	18° " 22° 204 "	2	48	Alt. temp.	0
Moldavica.....	50	3° " 7° 96 "	2	48	Cold, then warm	0
		17° " 21° 51 "				
Orthocarpus....	50	19° " 24° 35 "	0	49	Cold, then alt. temp.	0
Orthocarpus....	50	3° " 7° 96 "	4	46	Cold	0
		17° " 21° 51 "				

TABLE V—*Continued*

SPECIES	No. SEEDS USED	FIRST GERMINATING CONDITIONS	PER- CENT- AGE GERMI- NATION	No. SEEDS DRIED	GERMINATING CONDITIONS AFTER DRYING	PER- CENT- AGE DRIED SEEDS GERMI- NATING
<i>Pentstemon ryd- bergii</i> .....	50	3° to 7° 96 days 17° " 21° 51 "	14	43	Cold, then alt. temp.	19
<i>P. subglaber</i> ....	50	Alternating moisture 53 "	2	46	Cold, then alt. temp.	13
<i>P. subglaber</i> ....	50	3° to 7° 96 " 17° " 21° 51 "	6	47	Cold, then warm	0
<i>Polemonium</i> ....	50	22° " 29° 74 "	2	46	Cold, then alt. temp.	26
<i>Polemonium</i> ....	50	3° " 7° 96 " 17° " 21° 51 "	4	46	Cold, then warm	9
<i>Rumex</i> .....	50	Alternating moisture 21 "	0	50	Alt. temp.	86
<i>Sophia</i> .....	50	3° to 7° 96 " 17° " 21° 51 "	0	46	Cold, then warm	2
<i>Vicia</i> .....	25	3° " 7° 96 " 17° " 21° 51 "	8	22	Seed coat fired; cold then warm	100
<i>Vicia</i> .....	25	23° " 25° 26 "	0	16	Cold, then alt. temp.	0
<i>Viola</i> .....	50	Alternating moisture 21 "	0	37	Cold, then alt. temp.	0
<i>Ribes inebrians</i> ...	50	23° to 25° 35 " 3° " 7° 97 "	0	45	Alt. temp.	4
<i>Ribes inebrians</i> ...	25	3° " 7° 97 " 17° " 21° 51 "	0	24	Cold, then alt. temp.	17
<i>Ribes inebrians</i> ...	50	Alternating moisture 53 "	0	36	Cold, then alt. temp.	3
<i>Ribes inebrians</i> ...	50	22° to 29° 74 "	0	44	Cold, then alt. temp.	0
<i>R. montigenum</i> ...	50	23° " 25° 35 " 3° " 7° 97 "	0	33	Alt. temp.	0
<i>R. montigenum</i> ...	25	3° " 7° 97 " 17° " 21° 51 "	0	24	Cold, then alt. temp.	17
<i>Sambucus</i> .....	50	23° " 25° 35 " 3° " 7° 97 "	0	44	Alt. temp.	0
<i>Sambucus</i> .....	50	3° " 7° 97 " 17° " 21° 51 "	0	50	Cold, then warm	0
<i>Sambucus</i> .....	50	22° " 29° 74 "	0	48	Cold, then alt. temp.	0
<i>Symphoricarpos</i> ..	50	23° " 25° 35 "	0	43	Alt. temp.	0
<i>Symphoricarpos</i> ..	50	Alternating moisture 21 "	0	42	Cold, then alt. temp.	0
<i>Symphoricarpos</i> ..	50	18° to 22° 204 days	0	32	Cold, then alt. temp.	3

Of the five species which were kept from test C, the *Agropyron* seeds gave 40 per cent, *Hordeum* 94 per cent, and *Rumex* 86 per cent additional germination. *Symphoricarpos* and *Viola* seeds did not germinate under the new conditions.

## GERMINATION AFTER LONG PERIOD OF DRYING

Percentages of germination obtained with the seeds which had been dried for 105 days after having been in moist conditions for varying lengths of time are shown in table V. Nineteen of the 25 species and 28 of the 48 samples used gave varying percentages of germination.

## Discussion

The effect of alternate moistening and drying varied with the different species. In a few instances the effect of drying on the percentage of germination was negligible. Germination in some species was hastened and the percentage of germination increased by alternate moistening and drying, while with others germination was delayed and the percentage of germination decreased. Rapid and slow drying varied in their effect on germination in a number of the species studied. The behavior of the seeds depends upon the particular species, the rate of drying, and the time when drying takes place.

In general, the seeds of the species which germinated at room temperatures may be placed in five groups: (1) seeds in which alternate moistening and drying had no effect on percentage of germination; (2) seeds in which the percentage of germination was increased under conditions of alternate moistening and drying; (3) seeds in which the percentage of germination was decreased under conditions of alternate moistening and drying; (4) seeds in which germination was increased with rapid drying and decreased with slow drying; and (5) seeds in which germination was decreased with rapid drying and increased with slow drying.

While alternate moistening and drying had no effect on percentage of germination of *Bromus polyanthus*, rapid drying slightly delayed and slow drying hastened its germination (fig. 1).

Seeds giving increased germination have been noted in table III. The amount of increase usually varied with rapid drying and slow drying. However, both types of drying gave the same increase in percentage of germination in *Stipa lettermani*. The most pronounced effect of increase in germination was found in *Lepidium* (fig. 6) and *Rumex*, with rapid drying, and in *Artemisia* and *Rudbeckia* with slow

drying. The highest percentage of germination obtained in *Androsace* was with seeds which had been subjected to alternating moisture conditions with rapid drying.

With some seeds germination continued during the period of drying. In test *B* the seeds of *Poa interior* (fig. 2) began to germinate before the petri dishes were opened to dry, and continued to germinate during the period of drying. The seeds thus subjected to drying gave a slightly higher percentage of germination in a shorter period of time than the seeds which had continuous moisture. The behavior of the two lots of *Poa secunda* seeds was similar to, but lower in percentage, than *P. interior*. Germination also continued during the slow drying of the seeds of *Bromus anomalus*, *B. polyanthus*, *Artemisia*, and *Rudbeckia* (fig. 9).

In several species the percentage of germination secured with continuous moisture was lower at the temperatures used than the maximum germination obtained under other conditions (table I). Although temperature conditions were not favorable, percentages of germination approaching the maximum were obtained in *Achillea* and *Lepidium* under alternating moisture conditions with rapid drying, and in *Artemisia*, *Plantago*, and *Rudbeckia* with slow drying. In these species the effect of temperature was overcome by drying. In *Stipa lettermani* and *Rumex mexicanus* the effect of temperature was overcome by drying only in part. Under conditions of alternating temperatures *Rumex* seeds gave 100 per cent germination in 12 days. The effect of drying on *Rumex* was slowly to bring about the changes in the seed which were quickly induced by alternating temperatures. The foregoing results suggest that the effect of drying in these seeds is associated, at least in part, with the problem of oxygen supply, as CROCKER (3) found for *Xanthium*.

Drying decreased the percentage of germination in the seeds of several species. The amount of injury varied with the species and was influenced by the time and rate of drying. *Pseudocymopterus* (fig. 8) illustrates this effect. Slow drying of *Geranium* decreased germination slightly by hardening the seed coat of the few seeds which did not germinate quickly. Rapid alternations of moistening and drying only retarded the process of germination. The behavior



of *Geranium* agrees with the conclusions of DUVEL, who found that the retardation in germination after dry storage was caused by changes in the seed coat.

The knowledge regarding the nature of the changes taking place in the seeds of each particular species is far from complete. Drying may have produced changes in the seed coat or embryo, or in both seed coat and embryo. JOSEPH (19) observed that drying parsnip seeds during the storage period affected the embryo and not the seed coat. KIDD and WEST (21) concluded that the redrying of *Brassica alba* seeds overcame secondary dormancy, but DAVIS (8) could not secure any benefit from redrying *Ambrosia trifida* seeds in which secondary dormancy had been induced. JONES (18) observed that desiccation of after-ripened *Acer saccharum* seeds reduced seedling vigor, and that if desiccation continued the embryos failed to germinate. FLEMION (12) found that drying partially or entirely after-ripened seeds of *Sorbus aucuparia* developed a secondary dormancy which required longer to overcome than the original period of dormancy. She discovered too, that while the chief cause of dormancy was in the embryo, the seed coat also contributed to delay after the embryo had after-ripened. The effect of drying therefore differs with the different types of seeds.

#### IMPORTANCE OF TIME AT WHICH DRYING OCCURS

The time at which drying occurs is very important with some seeds. The time of drying in relation to the stage of development of the germinating seed was probably a factor in producing the results shown in table III, where rapid and slow drying gave opposite results. The effect of decreasing resistance to drying is seen in the slow drying of *Poa interior*, *P. secunda*, *Achillea* (fig. 4), and *Androsace* (fig. 5), and in both types of drying of *Chrysothamnus* and *Pseudocymopterus*. With rapid drying, which began late, *Bromus anomalous*, *Lupinus*, and *Rudbeckia* were injured. *Poa secunda* seeds with low viability did not withstand drying that started early in the germinative process. PFEFFER (27) calls attention to the fact that while resistant seeds can withstand drying and remoistening, seedlings are killed by drying, and resistance to drying decreases during germination.

Hastening of germination occurred in some seeds after slow drying (fig. 7) and in others after rapid drying (figs. 4, 8). In *Pseudocymopterus*, germination was hastened after the second period of rapid drying, although further drying decreased the percentage of germination. Such hastening and acceleration in germination of *Brassica alba* seeds after drying, as found by KIDD (20) and KIDD and WEST (21), may be due to the stage of development attained by the embryo while in a moist condition previous to drying, rather than to the influence of drying.

#### INFLUENCE OF CONDITIONS PREVIOUS TO DRYING

The influence of conditions previous to drying may be seen in the germination of some of the seeds which were dried for 105 days (table V). The three sets of *Melica* seeds were subjected to identical conditions during and after the period of drying. After drying and remoistening, followed by cold temperatures of 0° to 4° C. for 84 days, the seeds gave 94, 84, and 92 per cent germination respectively. Those seeds which had been placed at 19° to 24° C. for 35 days had not made as much development previous to drying as had the seeds which had been subjected to -1.5° to -2.5° C. for a part of the moist period, or those which had a longer period of moisture at the warmer temperatures. Although the percentage of germination was low, *Collomia* and *Ribes inebrians* also show the influence of conditions previous to drying. Here, as in *Melica*, the seeds which had been held at low temperatures previous to drying gave higher percentages of germination than those which had been given warm temperatures. Such results indicate that drying during the period of after-ripening had little, if any, effect upon the germination of seeds of *Melica*, *Collomia*, and *Ribes inebrians*.

#### EFFECT OF ALTERNATE MOISTENING AND DRYING AS SHOWN BY LATER TEST FOR VIABILITY

The effect of alternate moistening and drying was not shown on some seeds until after the period of alternating moisture conditions, when the seeds were placed in other conditions for germination. No germination was obtained during the time *Hordeum nodosum* and *Collomia linearis* seeds were subjected to alternating moisture con-

ditions with rapid drying. Apparently they were not harmed by such conditions and derived some benefit. In the test for viability following the period of alternate moistening and drying (table IV), *Hordeum* seeds from test *B* gave 74 per cent germination. While this was below the maximum obtained (table I), it was 50 per cent more than that secured from seeds under similar germinating conditions which had not previously been subjected to alternate moistening and drying. *Hordeum* seeds from test *C* which were dried for 105 days gave 94 per cent germination with alternating temperatures. (The percentage here is based on the original number of seeds and not on the number of seeds dried.) *Collomia* seeds which had been subjected to alternate moistening and drying in test *B* later gave the maximum germination obtained, 62 per cent, in a shorter period of time than seeds which had not been so treated.

When placed in new conditions for germination after the period of alternate moistening and drying, *Agropyron* and *Polemonium* each gave 20 per cent and *Pentstemon subglaber* gave 12 per cent additional germination. A few species gave less than 10 per cent germination.

Some seeds require a longer period for germination than was supplied to them in the test for viability following the period of alternate moistening and drying.

The effect of alternate moistening and drying on hardcoated seeds may be seen in *Lupinus parviflorus* and *Vicia americana*. In tests *A*, *B*, and *C*, the seed coats of both species were kept intact. Rapid alternations of moistening and drying retarded and decreased the germination of *Lupinus* somewhat, but had little effect on *Vicia*. Seeds of *Vicia* which were given continuous moisture gave no germination and seeds which were subjected to alternating moisture conditions gave only 5 per cent germination with either rapid or slow drying. After the period of alternate moistening and drying, the coats of the *Vicia* seeds which had not germinated in test *B* were broken by filing and the seeds again supplied with moisture. With this treatment 30 per cent additional germination was obtained, thus giving 35 per cent total germination for this set of seeds (table IV). At the same time, seeds which had not been subjected to alternating moisture conditions but in which the coats were broken by filing, gave 40 per cent germination.

Failure of seeds to germinate when placed in other conditions after 51 to 53 days of alternating moisture conditions, therefore, may have been due to injury from previous drying; to secondary dormancy produced by the drying, temperature, or moisture conditions; to lack of suitable germinating conditions; or in a few instances to lack of viability. Some seeds were injured by the alternate moistening and drying and others withstood repeated drying with little or no injury. Some of the *Poa secunda* seeds were not viable. Seeds from the second area had much lower viability than seeds from the first area. *Polygonum* seeds which failed to germinate and some seeds of *Pentstemon rydbergii*, *Acer grandidentatum*, and *Sambucus microbotrys* were found to be undeveloped. A few seeds molded during the period of testing, but many which failed to germinate still appeared sound. Since these seeds did not germinate with continuous moisture at room temperatures (22° to 29° C.) within the time employed, other conditions will have to be used for them before the effect of alternating moisture conditions can be determined. The seeds of a number of the species contain rudimentary embryos or embryos which require a long period of after-ripening before germination occurs.

#### EFFECT OF LONG PERIOD OF DRYING ON GERMINATION BEHAVIOR

Seeds which were dried for 105 days after a period of moistening also varied in their responses to germinating conditions (table V). Some may not have had suitable temperatures for germination, or the period may not have been long enough. One or more samples of each of four species, *Melica*, *Pentstemon rydbergii*, *Vicia*, and *Ribes montigenum*, gave higher percentages of germination after the long period of drying than the maximum germination obtained from seeds which were not so dried. Dried seeds of *R. inebrians* gave next to the highest germination obtained for the species. The behavior of *Melica* and *R. inebrians* seeds has been discussed in connection with the influence of conditions previous to drying. Of the two samples of *Vicia* seeds which were dried, those in which the seed coat had been broken by filing before they were remoistened gave 100 per cent germination. Those with coats intact gave no germination until later; when the coats were broken by filing, they also gave 100 per

cent germination. The long period of drying had no harmful effect on these seeds. The behavior of *V. americana* corresponds to the findings of MUNERATI and ZAPPAROLI for *V. segetalis*.

Some of the dried *Pentstemon rydbergii* seeds which failed to germinate were undeveloped, but 19 per cent germinated when remoistened. This was equal to 16 per cent of the original lot from which the dried seeds were taken. Previous to drying, 14 per cent had germinated, thus giving a total of 30 per cent for this lot of seeds, which was the maximum obtained for *P. rydbergii*. Thus germination was increased by drying the ungerminated seeds.

Little or no injury was shown by seeds of *Hordeum* and *Polemonium*. *Hordeum* seeds which had not been dried gave 100 per cent germination, and seeds which had been dried gave 96 to 97 per cent germination when subjected to alternating temperatures. *Polemonium* seeds which had been dried for 105 days gave 26 per cent germination while the maximum obtained in other tests was only 30 per cent.

Decreased germination of *Rumex* and *Orthocarpus* may have been due to injury or to secondary dormancy produced by drying, or by conditions which prevailed during the previous moist period. With alternating temperatures, *Rumex* seeds which had not been dried gave 100 per cent germination, but the seeds which had been dried after the period of alternating moisture conditions with slow drying gave only 86 per cent germination. *Orthocarpus* seeds gave 28 per cent germination when subjected to 0° to 4° C. for 87 days, but seeds which had been dried gave no germination although subjected to a temperature of 0° to 3° C. for a period of 91 days.

Lower percentages of germination than the maximum obtained with seeds which were not dried were obtained from the dried seeds of *Agropyron*, *Androsace*, *Chenopodium*, *Collomia*, *Frasera*, and *Gilia*. Besides *Orthocarpus*, which has been discussed, no germination occurred in the dried seeds of five other species, *Delphinium*, *Erythronium*, *Moldavica*, *Viola*, and *Sambucus*. Only one or two of the dried seeds of each species germinated in *Castilleja*, *Sophia*, and *Symphoricarpos*. Since several of these seeds have rudimentary embryos or embryos which require after-ripening, failure to germinate may have

been due to lack of suitable conditions for germination, or to secondary dormancy caused by drying rather than to injury or lack of viability.

#### EFFECT OF DRYING ON GERMINATION

The effect of drying on germination behavior is determined by the effect of drying on the seed itself. Drying seeds after they have absorbed water may affect them in one or more of the following ways:

1. Lack of sufficient moisture may arrest the development or growth of the embryo which precedes germination, until sufficient moisture is again supplied. When this is supplied development and growth proceed without injury and germination is only delayed.

2. Drying of the embryo at a critical stage in its growth, for example, when it is well developed just previous to or during the early stages of germination, when cell division is rapidly taking place, may injure it beyond the point of recovery and thus prevent further growth and germination when moisture is again supplied, as noted by KIDD, KIDD and WEST, and PFEFFER. This results in decreased germination.

3. Drying may bring about changes in the seed coat, making it more permeable to water or oxygen, or it may lessen the mechanical resistance of the coat to the developing embryo as found by LUDWIG and CROCKER (3, 4, 6). Such changes hasten and increase germination while the reverse of these changes in the seed coat may produce secondary dormancy, and thus delay or prevent germination (9).

4. Drying may bring about changes in the embryo which lower the vitality of the seed, decreasing germination, as found in the parsnip by JOSEPH and in the sugar maple by JONES (18); or it may produce secondary dormancy which delays and decreases germination until dormancy is again overcome, as in *Sorbus aucuparia* (12). Drying may bring about other changes in the embryo which hasten the development and germination of dormant seeds when moisture is again supplied. The behavior of some of the seeds used in this investigation indicates that the effect of drying may be on the embryo rather than on the seed coat. Further study is needed to determine definitely which part of the seed is affected.

The rate at which drying occurs and the time of drying often

determine whether it is harmful, beneficial, or negligible in its effect on germination. The amount of benefit or injury by alternate moistening and drying is difficult to determine, in some cases, until more is known about the normal germination behavior of the seeds. They vary considerably in their ability to withstand drying. Conditions which inhibit or delay the germination of seeds of one species stimulate and increase germination in another, and have no effect upon a third. The factors which bring this about can be determined only by further study of the individual species.

### Summary

1. The seeds used in this study comprise 9 grass, 26 weed, and 7 woody species of Utah range plants. Twenty-three of the 42 species studied gave little or no germination at temperatures of 22° to 29° C. Other conditions must be used for them before the effect of alternate moistening and drying can satisfactorily be determined.

2. The effect of alternate moistening and drying on the germination of seeds varies with the individual species. Of the 19 species which germinated at 22° to 29° C., alternate moistening and drying had little effect upon the germination of *Bromus polyanthus*. It increased the germination of *Stipa lettermani*, *Artemisia incompta*, *Lepidium densiflorum*, and *Plantago tweedyi*. It decreased the germination of *Geranium viscosissimum*, *Pseudocymopterus montanus*, *Chrysothamnus lanceolatus*, and *Stipa columbiana* (slightly).

3. Rapid drying had little effect upon the germination of *Poa interior*, *P. secunda*, and *Chenopodium album*, but increased the germination of *Achillea lanulosa*, *Androsace diffusa*, *Pentstemon rydbergii*, and *Rumex mexicanus*; in all of these species slow drying decreased the percentage of germination. Rapid drying decreased the germination of *Bromus anomalus*, *Lupinus parviflorus*, and *Rudbeckia occidentalis*, while slow drying increased the germination of *Rudbeckia* but had little effect upon *Bromus anomalus* and *Lupinus*.

4. Increased germination was usually accompanied by a hastening of the germinative process and decreased germination was accompanied by a retardation. In five species, however, *Poa secunda* no. 1, *Androsace*, *Plantago*, *Rudbeckia*, and *Rumex*, retardation was followed by increased germination.

5. Some seeds after they have absorbed water will withstand short periods of alternate drying and moistening, and some will withstand a long period of drying without injury. The time at which drying begins in relation to the stage of development of the germinating seed is a very important factor in determining whether drying is harmful or beneficial. The effect of drying is also influenced by the rate at which drying takes place.

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GREAT BASIN BRANCH STATION  
EPHRAIM, UTAH

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# RETARDED GERMINATION IN THE SEED OF HYPERICUM PERFORATUM CAUSED BY CALCIUM<sup>1</sup>

H. A. BORTHWICK

(WITH SIX FIGURES)

## Introduction

*Hypericum perforatum*, locally known as Klamath weed, is a serious pest in certain northern California range lands (6), where it has almost completely replaced the range forage. Studies of control methods, including both chemicals and range management, have been under way since 1930.

Early in the studies of conditions of seed germination, it was observed that germination takes place much more rapidly in distilled than in tap water, which resembles the soil solutions in which the seeds usually germinate.

## Methods

Seeds of *Hypericum perforatum* germinate as well, if not more readily, when submerged in water as when grown on moist cotton flannel.

A liquid germination medium has, for these experiments, certain distinct advantages. Petri dishes containing 25 cc. of liquid can easily be handled without danger of spilling, and since this amount of water or solution of salts is adequate for the tests, it was used in all cases. All tests were run in duplicate, and many in triplicate. Since the seeds are very small, 200 could be used in each dish, making 400 for each test. With this number the error of random sampling is reduced to a minimum.

This species does not germinate readily in the absence of light (3) unless nitrate is present in the germination medium (2). Germination in darkness proved considerably lower than in light. In both light and darkness, however, germination was far better in distilled than in tap water. For the purpose of these experiments, therefore,

<sup>1</sup> This investigation was conducted while the writer was at the University of California, Davis, California.

control of light appeared unnecessary provided the various lots of each experiment were subjected to the same light fluctuations. The petri dishes were grouped on a laboratory table where all were apparently equally exposed to the diffused light of the room.

Comparative tests were always run simultaneously so that all were subjected to the same temperature fluctuations.

TABLE I  
PERCENTAGE GERMINATION OF SEEDS IN TAP  
AND DISTILLED WATER

	PERCENTAGE GERMINATION			
	END OF 4 DAYS		END OF 7 DAYS	
	TAP WATER	DISTILLED WATER	TAP WATER	DISTILLED WATER
	2.5	58.0	48.2	88.5
	1.7	78.5	44.2	96.0
	4.0	62.0	44.2	93.2
	1.5	45.2	55.0	88.5
	2.5	46.5	41.5	92.0
	3.5	58.0	49.5	93.0
	2.0	61.5	50.5	88.7
	2.0	54.0	47.5	91.7
	4.0	69.5	40.0	92.5
	2.0	47.5	29.5	93.5
Mean..	2.6	58.1	45.0	91.8

### Experimental results

DIFFERENCES IN GERMINATION IN TAP AND DISTILLED WATER.—The data of table I are taken from the tap and distilled-water check lots from each of ten experiments. Each figure in the table is based on at least duplicate samples of 200 seeds each.

Figure 1 presents additional data, showing the rate of germination from the fourth to the ninth days inclusive. The points on each curve are based on germination data from 1200 or more seeds.

According to these data the effect of the tap water is mostly one of retardation. Figure 1 shows that the germination in tap water on the ninth day is only slightly higher than that attained in distilled

water on the fourth day. Presumably the germination in tap water might eventually have become as high as that in distilled had the tests been continued longer. Although germination was greatly retarded, there was little evidence that the tap water was toxic; the seedlings produced were healthy and vigorous.

The seeds sink much more readily after a few hours' soaking in distilled than in tap water, and they change color in tap water. The

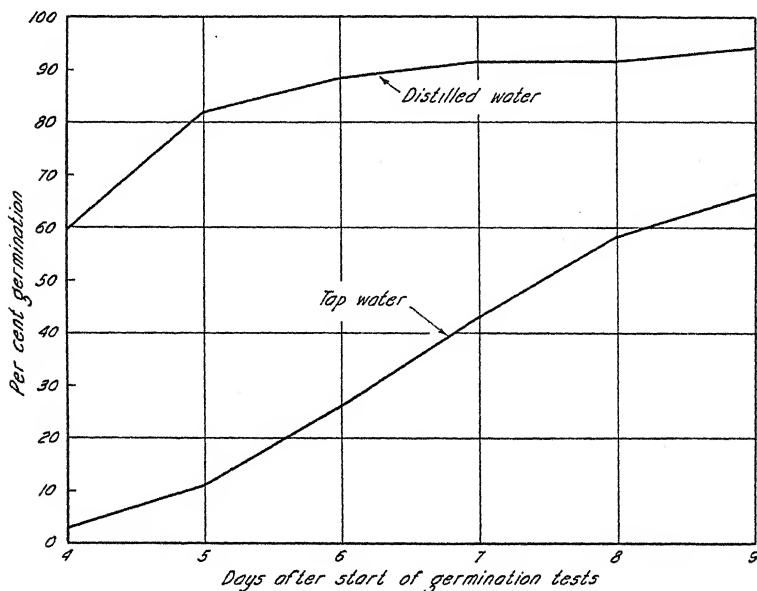


FIG. 1.—Rate of germination in tap and distilled water

dry seed, when mature, is brown, and when placed in distilled water it retains this color. In tap water, however, the seeds become jet black after a few hours of soaking.

Since a striking difference occurred in rate of germination in the two media, the effect of mixing the two was studied. The amount of tap water was varied by increments of 10 per cent from 0 to 100 per cent. As germination in all these mixtures was practically the same as in the tap-water check lots, evidently the critical concentration lay below 10 per cent tap water. A new series (fig. 2) was accordingly prepared with dilutions of 1, 3, 5, 7, 9, and 10 per cent tap water.

A mixture containing only 1 per cent of tap water distinctly retarded germination; a 3 per cent solution caused even greater retardation; and 5 per cent reduced the germination at the fourth day to about the same value as in pure tap water. At the end of seven days all lots showed much improvement, but especially the lots containing least tap water. No lot equaled the distilled water check at that time.

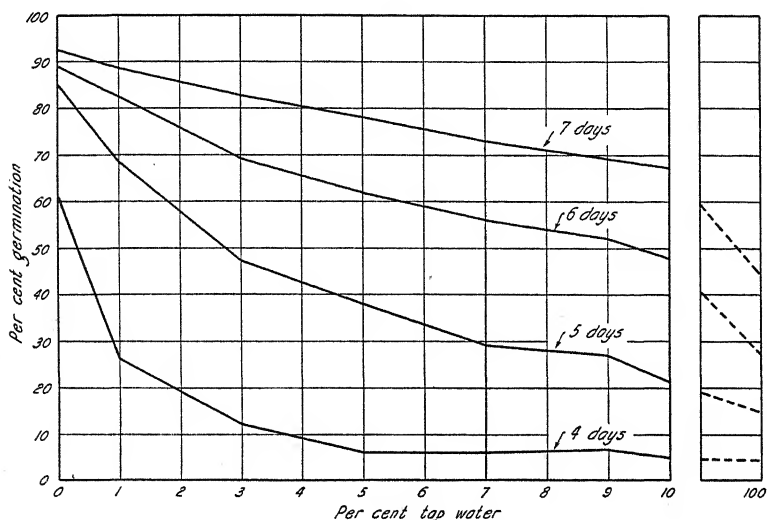


FIG. 2.—Germination in distilled water, tap water, and mixtures of the two containing 90 per cent or more distilled water.

In other experiments seeds were first placed in tap water and later changed to distilled. An extremely short immersion in tap water at the beginning of the experiment retarded germination materially (table II). Comparison of lots 1 and 2 of table II shows that one hour in tap water produced a retardation from which the lot did not fully recover until the seventh day of the test. Longer initial treatments with tap water produced even more pronounced delays.

POSSIBLE CAUSES OF LOW GERMINATION IN TAP WATER.—Attempts to explain the cause of these different germination responses suggested two lines of investigation: first, the effect on germination of various constituents of tap water; second, the effect of the pH of the medium. The tap water has a pH of 8.4 or higher, while the dis-

tilled water is slightly less than 6. This difference might account for the difference in rate of germination observed. On the other hand, the tap water is fairly high in magnesium, sodium, and bicarbonate ions, and conceivably these or other constituents might interfere with germination. Data are presented dealing with both these assumptions.

TABLE II  
RETARDING EFFECT ON GERMINATION CAUSED  
BY SHORT PERIODS IN TAP WATER

TREATMENT	PERCENTAGE GERMINATION			
	4TH DAY	5TH DAY	6TH DAY	7TH DAY
Distilled water check	17	59	78	81
Tap H <sub>2</sub> O 1 hour, then distilled H <sub>2</sub> O	5	39	72	82
" " 2 hours " " " "	3	33	68	79
" " 3 " " " "	4	32	67	83
" " 5 " " " "	3	35	69	82
" " 7 " " " "	4	28	66	82
" " 13 " " " "	2	24	58	73
" " 24 " " " "	3	23	55	78
" " 36 " " " "	4	26	59	78
" " 48 " " " "	3	20	58	80
" " 72 " " " "	1	12	39	64
Tap H <sub>2</sub> O check	1	8	24	37

EFFECT ON GERMINATION OF VARIOUS IONIC COMPONENTS OF TAP WATER.—The domestic water supply of the University Farm at Davis, California, is obtained from a deep well. The ions most abundant in it are present in the following approximate concentrations (milliequivalents per liter):

Na+ . . . . .	— 2.3	CO <sub>3</sub> <sup>=</sup> . . . . .	— 0.30
K+ . . . . .	— 0.0	HCO <sub>3</sub> <sup>-</sup> . . . . .	— 6.08
Ca++ . . . . .	— 1.4	Cl <sup>-</sup> . . . . .	— 0.38
Mg++ . . . . .	— 3.7	NO <sub>2</sub> <sup>-</sup> + NO <sub>3</sub> <sup>-</sup> . . . . .	— 0.05
		SO <sub>4</sub> <sup>=</sup> . . . . .	— 0.63
		H <sub>2</sub> AlO <sub>3</sub> <sup>-</sup> . . . . .	— 0.11

To study the effect of these various ions on germination, tests were run in solutions of various salts of Na, Ca, and Mg. In these solutions the concentration of each positive ion was made approximately equal to its concentration in tap water. Nitrate, sulphate, phosphate, chloride, and bicarbonate were the anions used. The results

indicated little difference between the sodium and magnesium salts; but the calcium salts gave consistently low results.

Inasmuch as single salts are sometimes toxic, solutions were then made up containing two salts each (table III). In those solutions which contained both sodium and magnesium, germination was high. When either of these two was used with calcium, however, the rate of germination was sharply reduced, although not to so low a value as that of tap water. These results confirm the observations made on germination tests run in single salt solutions, and indicate that primarily calcium causes the retardation.

TABLE III  
EFFECT ON GERMINATION OF VARIOUS IONIC  
CONSTITUENTS OF TAP WATER

CONTENT OF SOLUTION	PERCENTAGE GERMINATION			
	4 DAYS	5 DAYS	6 DAYS	7 DAYS
MgSO <sub>4</sub> *+NaCl <sub>2</sub> .....	22	58	80	86
CaSO <sub>4</sub> +NaCl.....	9	34	58	75
MgSO <sub>4</sub> +CaCl <sub>2</sub> .....	7	33	63	75
Tap water.....	1	5	13	29
Distilled water.....	34	69	81	88

\* In each solution, each positive ion used was present in a concentration of three milliequivalents per liter.

Since calcium salts retarded germination, their effect in different concentrations was studied. Calcium chloride was prepared in concentrations in which the calcium ranged from 0.02 to 200 times its concentration in tap water. Figure 3 shows the germination results from these solutions and from the tap and distilled water checks. It will be seen that the distilled-water check slightly surpasses even the most dilute calcium chloride solution used. The tap-water check, on the other hand, is slightly higher than a solution containing double the amount of calcium but considerably less than one containing 0.2 times as much calcium. These results show a close relationship between rate of germination and amount of calcium present in solution. They also check with the results obtained by diluting tap water with distilled (fig. 2).



As already shown, sodium salts have little influence on rate of germination. A pure sodium chloride solution containing 100 milliequivalents per liter gave better results than one of calcium chloride containing only 2.8 milliequivalents per liter. A series of mixtures of

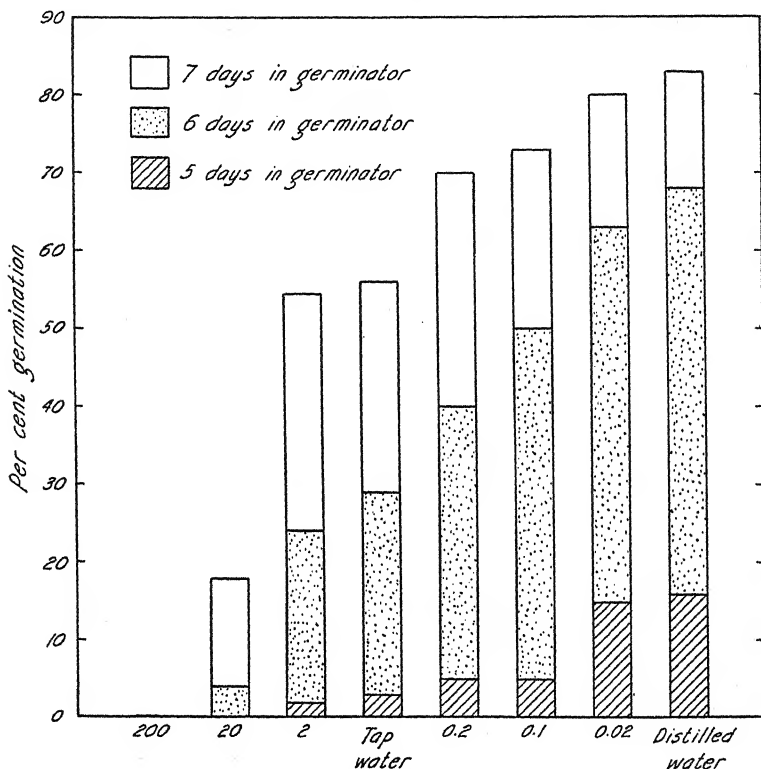


FIG. 3.—Germination in tap water, distilled water, and various concentrations of calcium chloride. Concentration of calcium chloride expressed in multiples of amount present in tap water.

the two salts was then prepared, in all of which the calcium chloride concentration was 2.8 milliequivalents per liter. The sodium chloride content was varied from 1 to 100 milliequivalents per liter. In all these the germination was as low as in pure calcium chloride of 2.8 milliequivalent concentration, indicating that this particular effect of calcium is not prevented by antagonism of sodium.

RELATION OF pH OF SOLUTION TO RATE OF GERMINATION.—The pH of the tap and distilled water mixtures previously discussed (fig. 2)

was not determined. A series (ranging from 0 to 100 per cent tap water) prepared later, however, showed that pH decreased with decreased concentration of tap water (fig. 4). Although data presented in figure 3 indicate that the concentration of calcium in these tap-water mixtures probably controls the germination results observed (figs. 2, 4), they do not prove that the pH variations are not in part responsible.

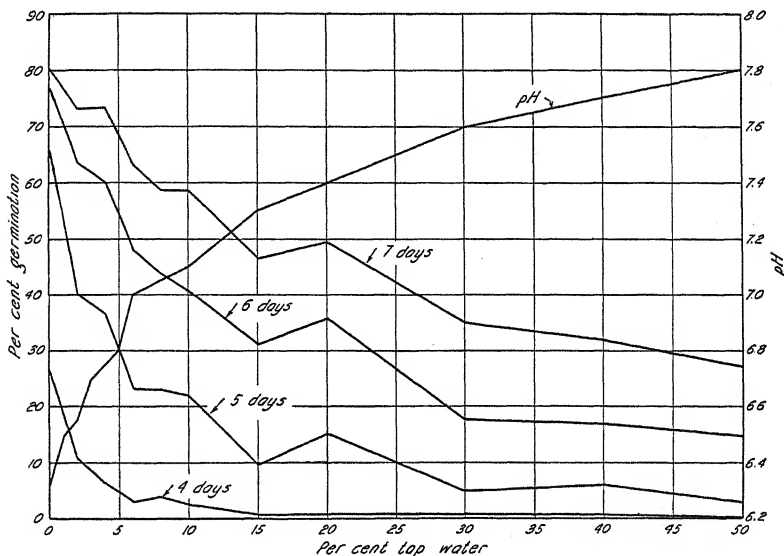


FIG. 4.—Relation of pH of various mixtures of tap and distilled water to germination in these mixtures.

A series of buffers was prepared containing the primary and secondary phosphates of sodium in total concentration equivalent to  $\frac{M}{300}$ . In this dilution these buffers gave a pH range from about 6 to 7.1, which covers the region in figure 4 where greatest differences in germination occur. The data (fig. 5) show that these variations in pH result in no significant difference in rate of germination. The buffers had no deleterious effect on the seeds; something other than pH causes the irregularities of germination in tap water.

This experiment was repeated with one modification. The buffer solutions were made up as before, but each solution was divided into two parts. One part was used as it was; to the other was added cal-

cium chloride at the rate of 2.3 milliequivalents of calcium per liter. In neither series was any significant difference found among the lots that could be attributed to differences in pH. The average germination for all lots in each series and those of the checks in tap and distilled water are plotted in figure 6.

While the germinations in phosphate buffers and in distilled water

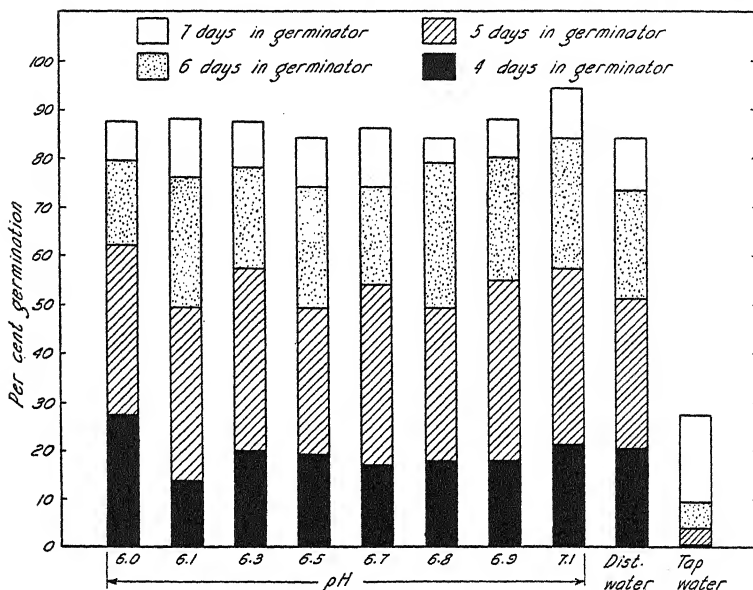


FIG. 5.—Germination in tap water, distilled water, and mixtures of primary and secondary sodium phosphates having a pH range from 6.0 to 7.1. Total concentration of salts in each phosphate buffer solution was  $\frac{M}{300}$ .

are practically parallel, a slight amount of calcium chloride added to the buffers reduces the germination to that of tap water. Measurements of the pH of buffers with and without calcium chloride showed no significant difference. The reduction in germination must therefore be attributed to the presence of calcium and not to a high pH. Although the tap water (pH 8.4) was much more alkaline than any of the buffers containing calcium (pH range 6 to 7.1), the germinations were almost identical.

WHY DOES CALCIUM RETARD GERMINATION?—To test the possibility that calcium in tap water may in some way modify the per-

meability of the seed coat or other cell layers to water, portions of the seed coat were dissected away to expose a part of the embryo. Although the minuteness of the seeds made dissection without injury impossible in many cases, many normal seedlings were produced in both tap water and distilled, and no significant difference in their

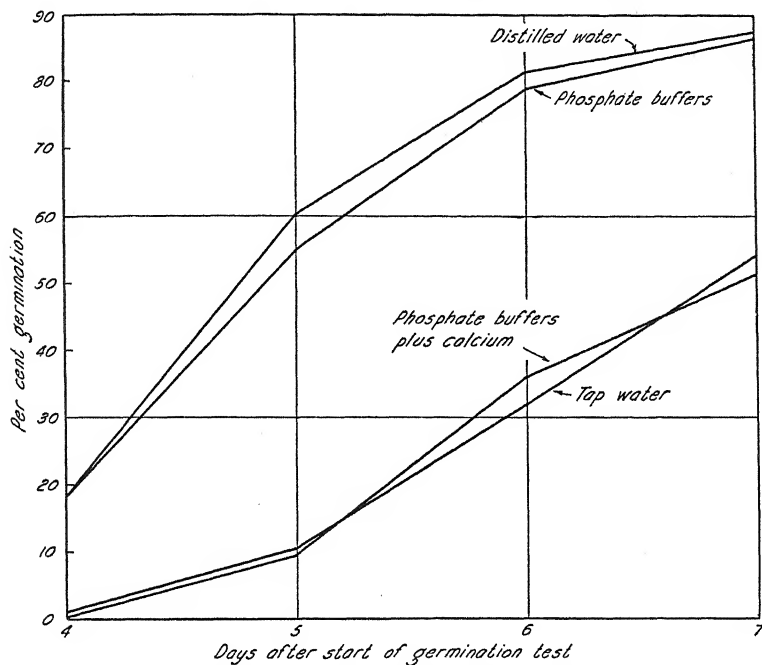


FIG. 6.—Rate of germination in phosphate buffers with and without calcium chloride compared with rate in distilled and tap water, respectively. Buffer curves based on mean germination in eight solutions having a pH range from 6.0 to 7.1. Calcium chloride series contained an amount of calcium approximately double that in tap water.

rates of emergence and growth could be detected. Evidently, then, the tap water does not inhibit germination if it has free access to the embryos; apparently its effect is upon seed-coat permeability.

The rate of water absorption from tap and distilled water respectively was next studied. Although the small size of the seed and the consequent large surface per unit weight make such determinations inaccurate, a significantly greater rate of absorption was nevertheless detected from distilled than from tap water. Two different methods were employed to remove the surface water from the seeds, and

by each of these a more rapid absorption from distilled water was demonstrated.

This difference in rate persists for a very short time. At the end of twelve hours the same amount of absorption occurs in both cases, and during the next few days no further intake is detectable by the methods employed. The short duration of a difference in rate of absorption made it improbable that this factor alone could account for the different rates of germination observed.

Lots of seed were placed in tap water the first day of the test and then transferred to distilled for the remaining six days. Other lots were placed in distilled water the first day, tap water the second day,

TABLE IV  
EFFECT ON GERMINATION OF VARIOUS ALTERNATIONS  
OF TAP AND DISTILLED WATER

TREATMENT	PERCENTAGE GERMINATION			
	4 DAYS	5 DAYS	6 DAYS	7 DAYS
Tap water continuously . . . . .	1	6	35	56
Distilled water continuously . . . . .	25	60	74	81
Distilled water on all but 1st day . . . . .	3	33	60	74
Distilled water on all but 2nd day . . . . .	5	24	49	70
Distilled water on all but 3rd day . . . . .	4	24	56	72

and distilled water again the remaining five days. Still others were kept in distilled water two days, then in tap water the third day, and distilled water again the last four days. Checks were run both in tap and in distilled water.

As table IV shows, all lots placed in tap water one day, whether on the first, second, or third day of the test, germinated at about the same rate; and this rate was intermediate between that of the two checks. Obviously the observed difference in rate of absorption during the first twelve hours is not responsible for the variability in germination rates.

This experiment, however, does not disprove the proposal that tap water alters the permeability of the coats to water, thus retarding germination. The structure of the seed coat indicates that much of the water absorbed during the first twelve hours of soaking is held in its outer layers. If that be true, the amount of water that actually

penetrates the seed coat to the embryo is small, and demonstration of differences in this rate in tap and distilled water would be difficult. Since there is a measurable difference in the initial rate of absorption by the whole seed, one might suspect a difference, difficult or impossible to detect, in the rate of movement of water from the seed coats into the embryo. The role of calcium in delaying germination, however, cannot be conclusively explained from the data presented.

### Discussion

The effect of calcium in retarding germination appears rather unusual; the writer is not aware of any other case where such small amounts of calcium have been shown to retard germination. In this connection several other kinds of seed were investigated and were found to be uninfluenced in their rate of germination by the presence of calcium.

TRUE (7) mentions that when a calcium salt is added to distilled water in concentration sufficient to make it osmotically equal to Potomac River water, the cells are in some way protected from the toxic effects of the distilled water. He found that sodium salts, on the other hand, fail to give this protection.

RUDOLFS (5) cites the work of CLAUDEL and CROCHETELLE (1), who found that alkaline substances with Ca or K as a base favor germination. He also mentions that MAQUENNE and DEMOUSSY (4) found calcium sulphate decidedly stimulating in the germination and growth of peas. RUDOLFS found that calcium nitrate affected adversely the germination and root length of nearly all seeds except corn. He presents germination data for only three kinds of seeds treated with calcium, however; and of these only one, white lupine, appears to show any detrimental effect ascribable directly to calcium. Furthermore, it is not conclusive that the difference of 16 per cent shown between the water check and the highest germination obtained after calcium nitrate treatment is significant, because only 50 seeds were used in each test, and the tests apparently were not duplicated.

Small concentrations of calcium may appreciably influence the rate of germination of *Hypericum* and possibly other seeds. Variations in rates of germination of *Hypericum* seeds in different soils and soil extracts do not appear to be correlated with the pH of the soil,

but may be associated with the concentration of calcium. In studying the effects of various toxic agents on germination, one should be aware of this effect and should not confuse it with the effects of the toxic substance.

### Summary

1. Germination studies with *Hypericum perforatum* seed showed that tap water retards germination as contrasted with the germination rate in distilled water. Mixtures of the two containing as little as 10 per cent tap water cause as pronounced germination delay as pure tap water. A relatively short period in tap water, followed by transfer to distilled water, also results in a definite retardation.

2. High alkalinity of the tap water and its ionic constitution are both possible causes of the effect observed. The data show that it is not a question of alkalinity, for similar results can be produced in solutions at or below the neutral point. The effects of the various ions present in tap water indicate that calcium causes the retarded germination.

3. No conclusive explanation is offered as to the method of operation of calcium in delaying germination. The data suggest that it alters the permeability of the coat to water.

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# STUDIES ON THE EMBRYOGENY OF THE SOLANACEAE. I<sup>1</sup>

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(WITH SIXTY-ONE FIGURES)

## Introduction

SOUÈGES (9) has studied the embryogeny in some of the genera of the Solanaceae. Considering the large number of genera belonging to this family, however, the literature on the subject appears to be meager. SOUÈGES holds that the embryogeny in the different members of the family follows the same plan of organization. Observations made by the writer on the embryology of some of the members of this family (2, 4) indicated a variation in the development of the embryo in *Physalis minima* L., which did not support SOUÈGES' generalizations. A detailed investigation was therefore undertaken to determine the range of variation in the development of the embryo in the same and different species of the Solanaceae. Such a study may be helpful in discovering the phylogenetic relationship between different members of the Solanaceae and the affinities of this family to others. The present paper is therefore only a part of the contemplated work on this subject.

**MATERIAL AND METHODS.**—The material was obtained from *Physalis minima* L., *Withania somnifera* Dun., *Nicotiana plumbaginifolia* Viv., and *Petunia nyctaginiflora* Juss., grown in the University experimental garden during the year 1931-32. Fruits of different sizes at different stages of development were dissected from healthy plants during bright days, and were killed and fixed, chiefly in Licent's fluid, between 12:30 and 2 P.M. To facilitate penetration, the air was removed from the fluid and the tissue with an exhaust pump, immediately after fixation. The material was then treated as necessary for the paraffin method and sections were cut 6 to 12  $\mu$  thick. Heidenhain's iron-alum haematoxylin with or without a counter stain was employed.

<sup>1</sup> Contribution from the Department of Botany, Calcutta University.



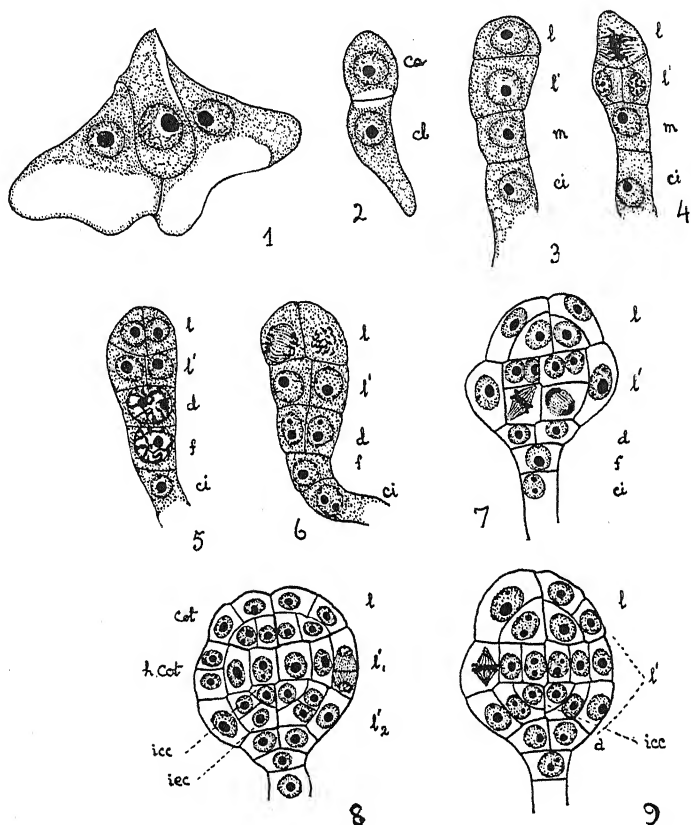
### Observations

The time required for the first division of the zygote varies in different genera of the Solanaceae, but the zygote always rests for some time before commencing activity. The endosperm nucleus divides many times, forming a cellular endosperm tissue, before the zygote divides. FERGUSON (6) first showed in *Petunia* that the primary endosperm nucleus divides to form a two-celled endosperm tissue, even prior to discharge of the sperms from the pollen tube. Similar observations have been made by the writer (3) in *Lycopersicum esculentum* Mill. as well as in *Petunia nyctaginiflora* Juss. No trace of synergids has been noted during the first division of the zygote. A quadrant of four endosperm cells is generally cut off by two successive longitudinal divisions of an endosperm cell at right angles to each other; they surround the distal end of the zygote and supply nutrition to it (fig. 1). Development of the embryo begins with elongation of the zygote. The first division is always transverse. The two daughter cells then divide transversely, forming a four-celled linear proembryo (figs. 2, 3, 11, 25-28). The apical cell of the two-celled proembryo may divide either before or after the basal cell divides (figs. 26, 27). According to SOUÈGES (9), the division of the apical cell of the two-celled proembryo generally takes place first. From the present account it will be seen that the basal cell not infrequently divides before the apical cell. In some cases the proembryo begins to bend from the two-celled stage, instead of becoming linear, and looks like an arch in the four-celled stage (fig. 29).

For convenience, the specific cells of the four-celled proembryo are designated as follows: the cell nearest the micropyle is termed *ci*; the next cell above it, *m*; the next cell above this, *l'*; and the distal cell, *l*. The first products of *l* are *l<sub>1</sub>* and *l<sub>2</sub>*; that of *l'* are *l'<sub>1</sub>* and *l'<sub>2</sub>*; that of *m* are *d* and *f*; and the products of *ci* are *n* and *n'*. These symbols are like those followed by SOUÈGES (9).

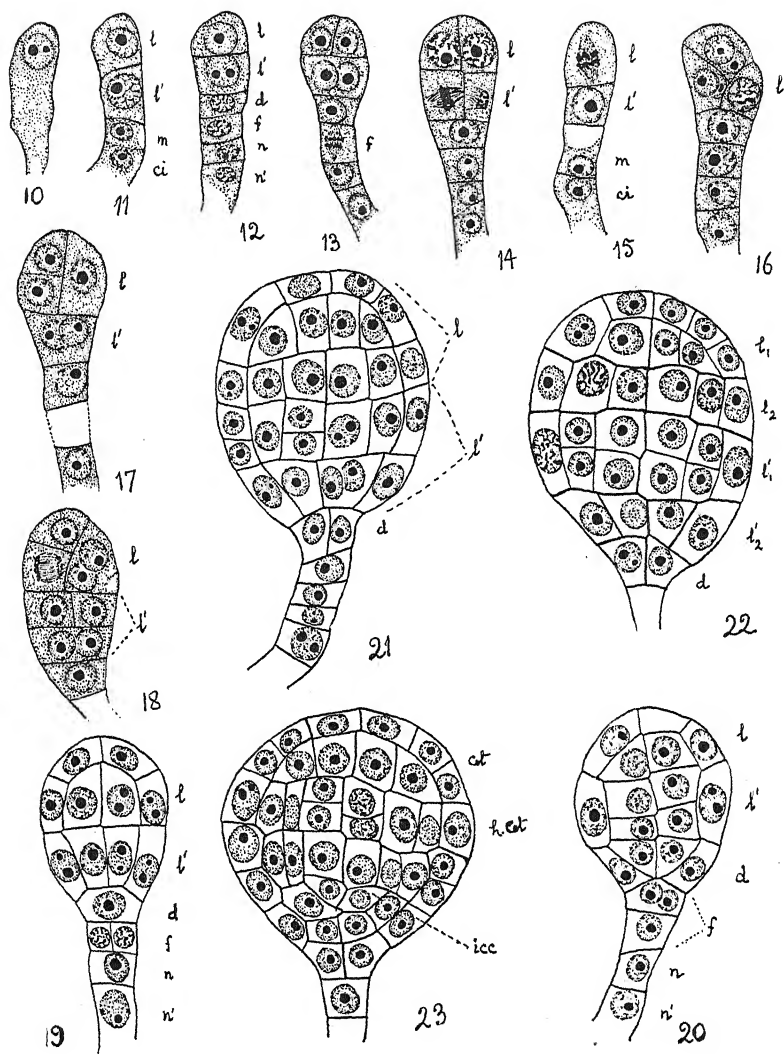
From the four-celled stage, subsequent development generally varies in the different (and sometimes even in the same) species. Four principal types of development have been traced during this investigation, and these four types have all been observed in *Phy-salis minima*.

The general mode of development of the embryo in *Nicotiana plumbaginifolia*, *Petunia nyctaginiflora*, as well as in some embryos of *Withania somnifera* and *Physalis minima*, corresponds to the



FIGS. 1-9.—Stages in development of embryo in *Petunia nyctaginiflora*. Figure 1 is a longitudinal section through micropylar end of embryo sac, showing two out of the four endosperm cells covering the zygote; they look like synergids.  $\times 600$ . (*icc*, initials of central cylinder; *iec*, initials of root tip; *cot*, cotyledons; *h.cot*, hypocotyl; explanation of other symbols is given in the text).

"*Nicotiana* type" of development already described (9). In these species the apical cells *l* and *l'* first divide longitudinally, whereas the divisions of *m* and *ci* are transverse. An eight-celled proembryo six cells long is thus produced (figs. 13, 32). Sometimes the longitu-

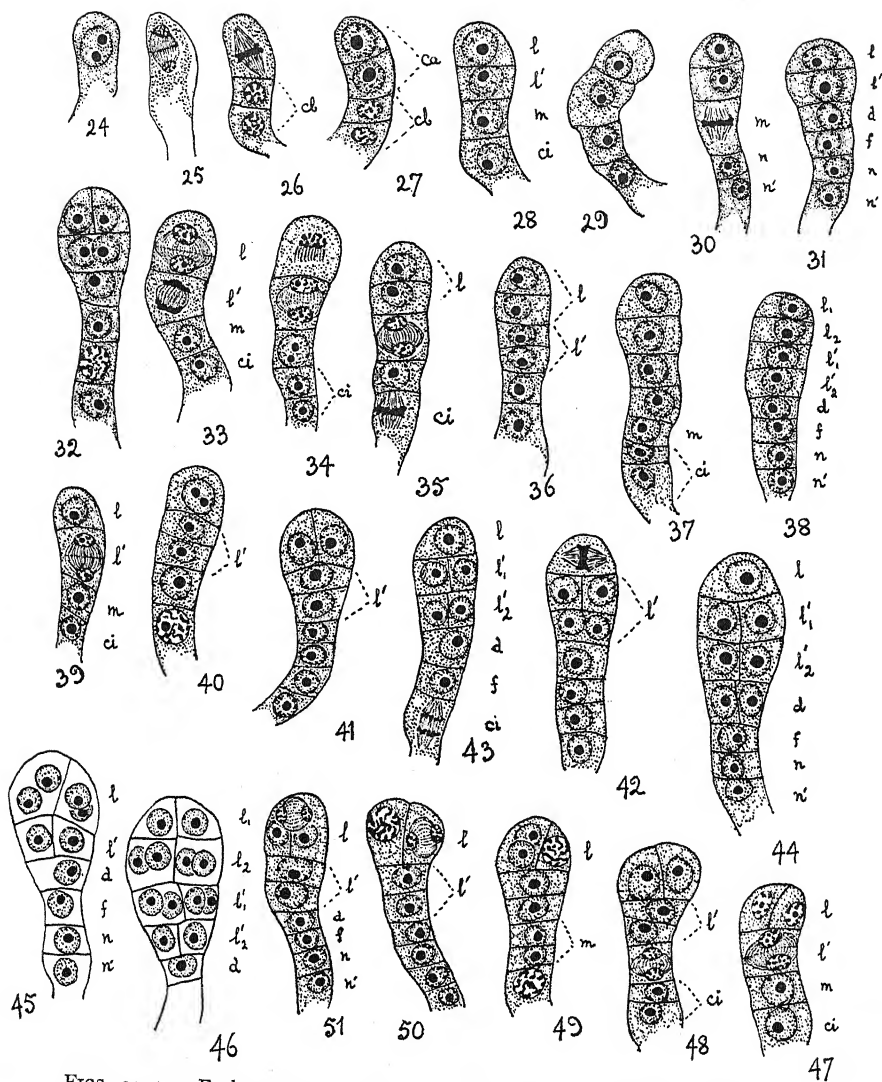


FIGS. 10-23.—Stages in development of embryo in *Withania somnifera*: Figs. 11-13, development of proembryo to six and eight-celled stage; fig. 14, formation of octant stage; figs. 15-18, aberrant type of development of proembryo; fig. 20, differentiation of initials of hypocotyl and root tip from inner cells of *l'*.  $\times 600$ .

dinal divisions in  $l$  and  $l'$  take place before the cells  $m$  and  $ci$  divide transversely, whereby a six or seven-celled proembryo four or five cells long is produced (fig. 5). It has been found, as previously noted by SOUÈGES, that the cell  $l'$  generally divides earlier than the cell  $l$  (fig. 4). Following the first division of  $l$  and  $l'$  they divide longitudinally, forming two tiers of four cells each (figs. 6, 14, 45). The next division in each of these resulting eight cells is periclinal, forming an outer and an inner cell (fig. 19). The outer cells form the dermatogen. These last periclinal divisions have sometimes been observed to take place earlier in the division of  $l'$  than of  $l$ . The four inner cells of  $l'$  then divide transversely, separating the initials of the hypocotyl above and of the radicle below (figs. 20, 53, 56).

In the second type of development, all the four cells  $l$ ,  $l'$ ,  $m$ , and  $ci$  divide transversely before any longitudinal walls appear in  $l$  and  $l'$  (figs. 33-38). This type of eight-celled proembryo is commonly met with in *Physalis minima*. SOUÈGES (9) occasionally found similar proembryos in *Solanum sisymbirifolium*. Further transverse divisions of  $l_1$  and  $l_2$  or  $l'_1$  and  $l'_2$  have not, however, been observed during the present study. The first three or four cells farthest from the micropylar end of the proembryo then divide longitudinally, forming an eleven or twelve-celled proembryo eight cells long. The products of these apical cells then divide again longitudinally. As a result three tiers of four cells each are formed at the distal end of the proembryo (figs. 46, 52). The next division in each of these cells is periclinal, forming an outer and an inner cell (fig. 55), the outer one forming the dermatogen. The transverse divisions leading to the separation of hypocotyl from radicle do not necessarily take place in this type of development.

In the third type of development, the cells  $l'$ ,  $m$ , and  $ci$  divide transversely but the first division of the cell  $l$  is longitudinal (figs. 39-41). The longitudinal division of  $l$ , however, generally takes place after the transverse divisions in  $l'$ ,  $m$ , and  $ci$  are completed, forming an eight-celled proembryo seven cells long. Here also the three distal cells divide longitudinally, twice in succession, forming three tiers of four cells each. Sometimes the longitudinal division in  $d$  is delayed. Subsequent divisions follow the same sequence as in type two (figs. 42-44, 54).

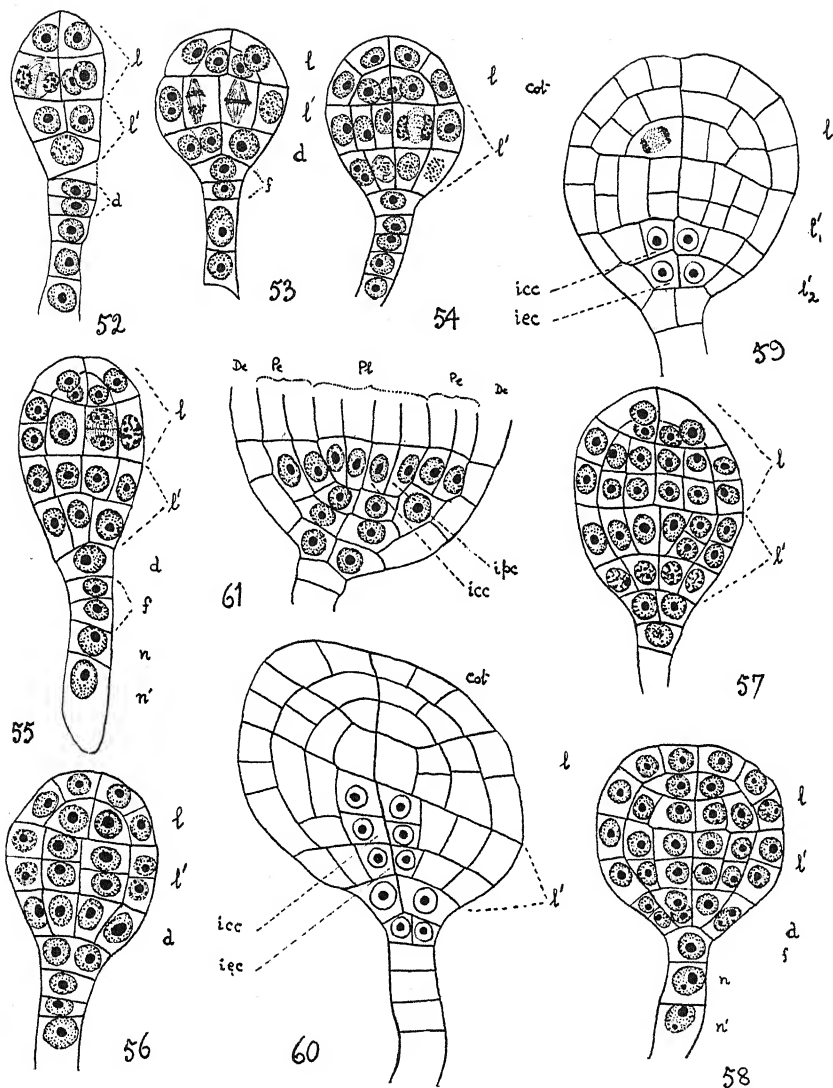


FIGS. 24-51.—Early stages in development of embryo in *Physalis minima*: Figs. 24-29, development of four-celled proembryo from zygote; figs. 30-32, development of six and eight-celled proembryo according to first type of development; figs. 33-38, development of eight-celled linear proembryo according to second type of development; figs. 39-41, development of proembryo according to third type of development; figs. 42-51, development of proembryo following fourth type of development; figs. 43-46, later stages of proembryo according to different types of development.  $\times 600$ .

In the fourth type of development, the distal cell  $l$  divides longitudinally in the four-celled stage (fig. 47). The cell  $l'$ , and also  $m$  and  $ci$ , later divide transversely as in the third type of development, forming an eight-celled proembryo seven cells long (fig. 48). The difference in the subsequent divisions of these proembryonal cells from the previous type is that, before any longitudinal division takes place in  $l'_1$  and  $l'_2$ , the first products of  $l$  again divide longitudinally and each of the four cells thus formed divides transversely, forming two tiers of four cells each. The last transverse division is assumed, however, for as soon as this division is completed it becomes very difficult to say whether the apical cells have divided longitudinally twice in succession or a single apical cell has first divided longitudinally twice in succession followed by a transverse division. A critical examination of the sequence of divisions of the individual cells from the four-celled stage, however, leaves no doubt that some of the embryos follow this latter type of development (figs. 49-51).

The octant stage here is thus produced from the activity of a single apical cell,  $l$ . BORTHWICK (5) has described similar aberrant types of embryo development in *Daucus*. Similar divisional stages, as have been shown in the drawings of BORTHWICK, have, however, not been observed. By this time the cell  $l'$  divides longitudinally and later by another longitudinal division forms a plate of four cells, completing the dermatogen at the radicular apex of the embryo, following a periclinal division.

Differentiation of the initials of the central cylinder from the root cortex, that is, differentiation of the periblem and the plerome at the radicular apex of the embryo, takes place differently in some of the different genera studied. In *Petunia* the origin of the initials of the central cylinder is the result of an oblique division of the inner cell  $l'_2$  (figs. 7, 9). In *Nicotiana*, *Physalis*, and *Withania* the initials of the central cylinder are differentiated by transverse or longitudinal divisions of  $l'_1$  or  $l'_2$  (figs. 23, 57-60). In some embryos differentiation of the initials of the pericycle takes place earlier, owing to longitudinal divisions of the initials of the central cylinder (fig. 61). In exceptional cases differentiation of the initials of the central cylinder



FIGS. 52-61.—Later stages in development of embryo in *Physalis minima* ( $ipc$ , initials of pericycle;  $de$ , dermatogen;  $pe$ , periblem;  $pl$ , plerome).

has been found to be due to slightly oblique divisions of the products of  $l'$ . In the fourth type of development of the embryo, already described, the initials of the central cylinder can originate from products of either  $l_2$  or  $l'_2$ .

The relationship of the different organs of the embryo to that of the four cells in the four-celled stage, however, is very different in the four types of embryo development. SOUÈGES (7, 8) considers

TABLE I

ORIGIN OF PARTS OF MATURE EMBRYO FROM CELLS OF FOUR-CELLED PROEMBRYO IN THE FOUR TYPES OF EMBRYO DEVELOPMENT

INDIVIDUAL CELLS OF FOUR-CELLED PROEMBRYO	PARTS OF MATURE EMBRYO DERIVED FROM EACH CELL OF FOUR-CELLED STAGE			
	TYPE I	TYPE II	TYPE III	TYPE IV
$l$ .....	Cotyledons	Cotyledons and hypo- cotyl	Cotyledons	Cotyledons, hypocotyl, and root tip; or cotyle- dons and hy- pocotyl
$l'$ .....	Hypocotyl and root tip	Root tip, root cap, and up- per part of suspensor	Hypocotyl and root tip	Root cap and upper part of suspensor
$m$ .....	Root cap and upper part of suspensor	Suspensor	Root cap and upper part of suspensor	Suspensor
$ci$ .....	Suspensor	Suspensor	Suspensor	Suspensor

this to be very important in the study of the embryogeny of flowering plants. The differences are shown in table I.

The suspensor of the mature embryo in all the species studied consists of a variable number of cells ranging from five to ten. In *Cestrum diurnum* L., observations regarding the embryogeny of which have not yet been completed, the suspensor is massive and is unlike that found in any other species of the Solanaceae.



The phenomena of polyembryony, under natural conditions, have already been reported by BANERJI and BHADURI (1) in a number of species of the Solanaceae.

### Discussion

From a comparative study of the embryogeny of different plants, SOUÈGES (7-11) has concluded that the arrangement of the cells in the four-celled stage and the subsequent behavior of these four cells in the formation of the different parts of the embryo are important essentials in the study of the embryogeny of angiosperms. He has two main types of arrangement of the four cells in the four-celled stage. In the first type two distal cells *l* and *l'* lie side by side and the proembryo is three cells long. This type, commonly found in flowering plants, is a constant feature of the Liliaceae, Ranunculaceae, Cruciferae, Compositae, etc. In the second type a linear proembryo four cells long is formed. This type is common in the Solanaceae, Umbelliferae, Rubiaceae, Scrophulariaceae, etc. From the subsequent behavior of these four cells SOUÈGES has further concluded that each cell of the four-celled proembryo gives rise to a definite region of the mature embryo. This character is mostly common for the different members of the same family or group of plants. It differs, however, in different families and groups of plants. He also holds that the way in which the different parts of the mature embryo are formed from the individual cell of the four-celled stage is very significant in indicating plant relationships.

The study of the embryogeny in the Solanaceae was first made by TOGNINI (12), who described only a few stages in the development of the embryo in *Solanum tuberosum*, *Datura stramonium*, *Physalis edulis*, and *Atropa belladonna*. SOUÈGES (9) studied a number of species belonging to this family and concluded that his generalizations hold good for the entire family. He observed that a linear proembryo is always formed. The present investigation supports this generalization. From the present study, however, it will be seen that the other conclusion of SOUÈGES (9), that definite parts of the mature embryo are always formed from a particular cell of the four-celled proembryo, is not tenable. According to his generalizations, the cotyledons are derived from cell *l*, the hypocotyl and the

root tip from cell *l'*, the root cap and upper part of the suspensor from cell *m*, and the remaining part of the suspensor from cell *ci*. Table I, however, shows that this generalization is incorrect; at least the writer found it so in the case of *Physalis minima*, in which the same part of the embryo originated from different cells of the four-celled proembryo. From the drawings of SOUÈGES (9) it appears that he also observed some stages in *Solanum sisymbirifolium* similar to those seen during the present study. Possibly SOUÈGES has misinterpreted some of the advanced stages of embryo development. In the Umbelliferae SOUÈGES (10, 11) also found a variation in the development of the proembryo in *Carum carvi*, similar to that observed in *Physalis minima*. He concluded, however, that the organs of the mature embryo in these cases also develop from a particular cell of the four-celled proembryo. BORTHWICK (5), on the contrary, has made it clear that this generalization of SOUÈGES regarding the embryogeny of the Umbelliferae also fails in the case of *Daucus carota*. BORTHWICK also thinks that SOUÈGES might have misinterpreted some of the advanced stages of embryo development in *Carum*.

It will be seen from the present observation that the method of origin of the different parts of the mature embryo varies in different and sometimes even in the same species of Solanaceae. From the observations of SOUÈGES as well as from the present study, it will be further seen that the method of differentiation of the initials of the central cylinder is generally fixed for such genera as *Nicotiana*, *Hyoscyamus*, *Atropa*, *Petunia*, etc. In *Solanum*, *Withania*, and *Physalis* a variation in the origin of the initials of the central cylinder and the different parts of the mature embryo is very common.

From a comparative study of the embryology and of the morphological resemblances existing in the Umbelliferae and Solanaceae, SOUÈGES (11) has concluded that a close phylogenetic relationship exists between these two families. There exists much controversy as to the phylogenetic position of the Umbelliferae. SOUÈGES concluded that these two families should be put in the same order, Solanales. The observations of BORTHWICK (5) have, however, weakened SOUÈGES' conclusion. From the present study it will

be seen that the embryogeny of *Physalis minima* is very similar to that observed by BORTHWICK in *Daucus carota*, and that the variation in the embryogeny of the two families appears to be the same. But from the present state of our knowledge regarding the embryogeny of these two families it will not be safe to accept SOUÈGES' theory until further data accumulate on their embryology.

### Summary

1. In the light of SOUÈGES' theory, the development of the embryo from the zygote has been studied in *Physalis minima*, *Withania somnifera*, *Nicotiana plumbaginifolia*, and *Petunia nyctaginiflora*.

2. The generalization made by SOUÈGES, that the four cells of the four-celled proembryo always give rise to the same part of the mature embryo, seems to be untenable in the light of this investigation.

3. The variation in the development of the embryo in the Solanaceae and Umbelliferae appears to be the same.

The writer desires to express his thanks to Mr. I. BANERJI for his helpful suggestions and constant encouragement throughout this investigation.

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## STUDIES ON AMBROSIA. II: EFFECT OF CERTAIN ENVIRONMENTAL FACTORS ON FLORAL DEVELOPMENT OF AMBROSIA ELATIOR

KENNETH L. JONES

(WITH SIXTEEN FIGURES)

This paper is a report on some observations and minor experiments made during the past six years in the course of genetical studies on the floral types of the ragweed, *Ambrosia elatior* L., recently published (4). Plants of this species may be conveniently placed into four categories or forms, based on breeding behavior, and these are briefly listed because the environmental effect depends upon the form used.

### MONOECIOUS FORMS

FORM 1.—This is the well known form, widely distributed in nature, in which the racemes are staminate (fig. 1) and the pistillate heads occur in leaf axils. It breeds true, when used as the female parent, regardless of the form of the male parent, which does not affect the floral type in this species.

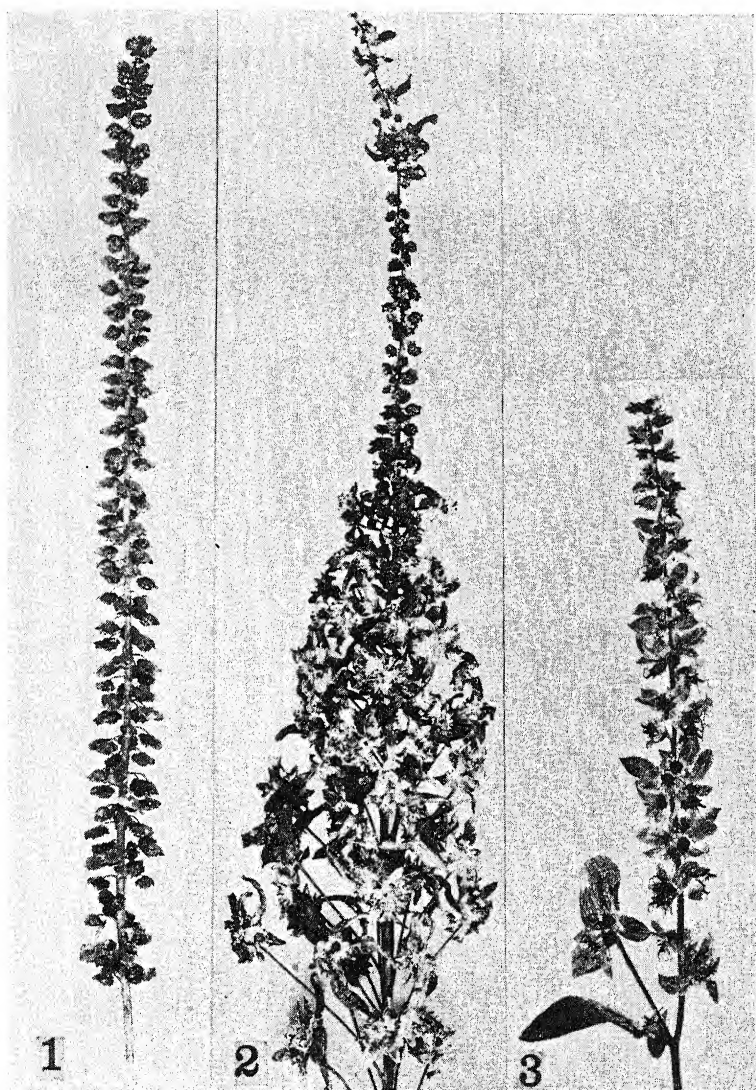
FORM 2.—Identical in appearance to form 1, but does not breed true.

FORM 3.—Racemes contain both staminate and pistillate flowers (fig. 2); does not breed true, but produces more pistillate offspring than does form 2.

### PISTILLATE FORM

Racemes pistillate (fig. 3); does not breed true, but produces more pistillate offspring than do the monoecious forms.

It is to be noted that only monoecious form 1 breeds true, and that there are no secondary characteristics to distinguish the forms prior to anthesis, so that it is usually impossible to determine environmental effects on floral development of individual plants. The method employed was to compare ratios of forms in treated cultures with a control grown from seed derived from the same plant.



FIGS. 1-3.—Fig. 1, staminate raceme of monoecious form 1 or 2; fig. 2, androgynous raceme of monoecious form 3; fig. 3, pistillate raceme of pistillate form.

## Results

The effects of waterlogged soil, photoperiods of 20 and 24 hours, and the removal of plumules were studied. The results are recorded in table I. The data indicate that the role of the environment on the floral development depends upon the form used, since the offspring of forms 1 and 3 were not altered by any of the treatments, whereas form 2 and P (pistillate) were affected.

TABLE I

FORM	TREATMENT	No. SEEDLINGS	No. PLANTS TO FLOWER	PERCENTAGE		
				1-2*	3	P
1	Control.....	100	100	100	0	0
1	Waterlogged.....	100	98	100	0	0
1	20 hour photoperiod...	98	40	100	0	0
1	24 hour photoperiod...	100	60	100	0	0
1	Plumules removed.....	60	47	100	0	0
2	Control.....	209	174	67.8	24.1	8.0
2	Plumules removed.....	99	90	53.3	32.2	14.4
3	Control.....	163	145	27.5	34.4	37.9
3	Waterlogged.....	100	92	28.2	33.6	38.0
P	Control.....	201	170	1.7	40.5	57.6
P	Waterlogged.....	100	93	8.6	13.9	77.4
P	20 hour photoperiod...	93	22	13.6	4.5	81.8
P	24 hour photoperiod...	100	58	5.1	25.8	68.9

\* Monoecious forms 1 and 2 are identical in appearance.

The instances of altered ratios require comment. Mutilation, pruning, and the removal of floral buds have often been reported to produce changes in floral development, for example HIGGINS (3), MURNEEK (5), and PRITCHARD (6). The results secured from pruning mature plants of *A. elatior* were unsatisfactory; form 3 alone was altered, and the results depended upon the distribution of the different heads in the racemes. The removal of plumules from seedlings of plants derived from form 2 increased the percentage of pistillate plants. This treatment was not a means of introducing a single variable, of course, for it entirely changed the plants to a dichotomous habit, and arrested their development so that anthesis occurred 2-3 weeks later than the controls which consequently grew under different conditions of temperature, light, and humidity. It is significant

that, in spite of the altered ratios, plants of forms 1-2 predominated and pistillate plants were least abundant in both the control and the culture in which the plumules were removed.

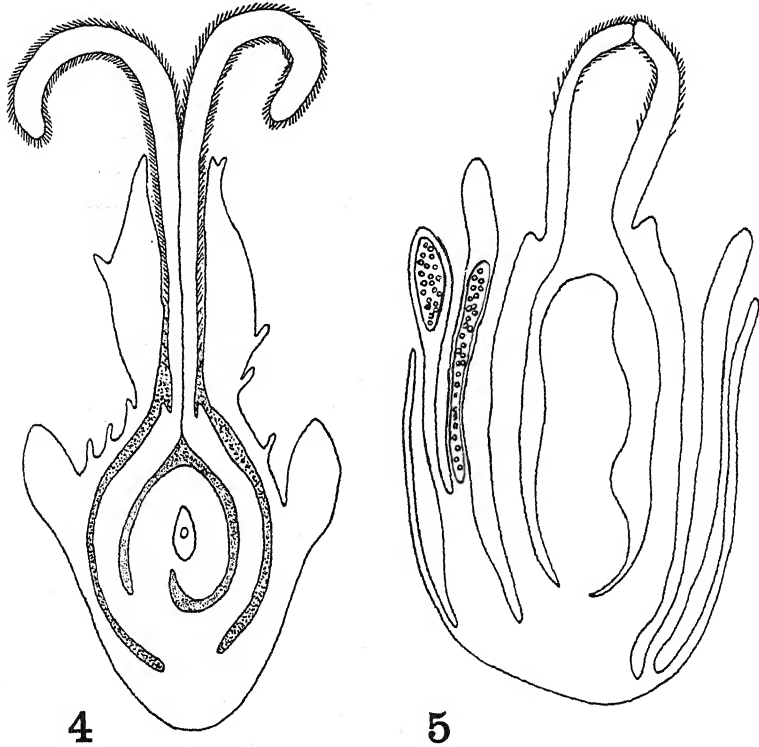
The offspring from a pistillate plant gave very dissimilar ratios when subjected to various treatments, although there was never a differential swing toward the staminate or pistillate condition, since an increase occurred both in the predominantly staminate forms 1-2 and in the pistillate form. The writer had no intention of investigating the effect of waterlogged soil on floral development, but secured these data in testing the effect of a high concentration of  $\text{Ca}^{++}$ ,  $\text{K}^{+}$ ,  $\text{PO}_4^{=}$ , and  $\text{NO}_3^{-}$  ions. The control culture, kept drenched with rain-water, gave practically the same deviation from the control in the field as those in which the soil was kept saturated with the foregoing nutrients, so that the effect was apparently the result of anaerobic conditions in the waterlogged soil.

The photoperiod is a significant factor in the growth of *A. elatior*; for example, plants (from any form) which flower during the short days of December or January are very dwarfed, and usually fail to produce racemes. They may form a few staminate heads at the terminal growing point, but often produce only lateral flowers, which are always pistillate. Such pistillate plants, in a culture which should be monoecious, cannot be regarded as "sex reversals" since pistillate flowers have not replaced staminate flowers. GARNER and ALLARD (2) grew six plants of *A. elatior* under a photoperiod of seven hours from June 3 to July 1, when pollination began. The plants were returned to normal light conditions and "blossomed a second time during the last week in August, which is also the time of blossoming of the original controls and of ragweed growing in the field. It may be noted, however, that while the original growth produced staminate spikes as well as pistillate flowers in the usual manner, the second growth produced pistillate flowers almost exclusively." Long photoperiods delay flowering; for example, at 20 hours pollination began November 10, whereas the control pollinated August 15. It is possible, of course, to assume that the ratios shifted because form 3 constituted the major portion of the large group of plants which continued to vegetate and died without flowering.

It has been found that racemes, even of form 1, may be modified



if the photoperiod is increased after they have begun to form. Figure 16 shows an extreme case in which a raceme from a form 1 plant developed in turn two pistillate heads, several staminate heads, several pistillate heads some of which were subtended by pinnatifid leaves, and finally staminate heads. This is the only instance in this species

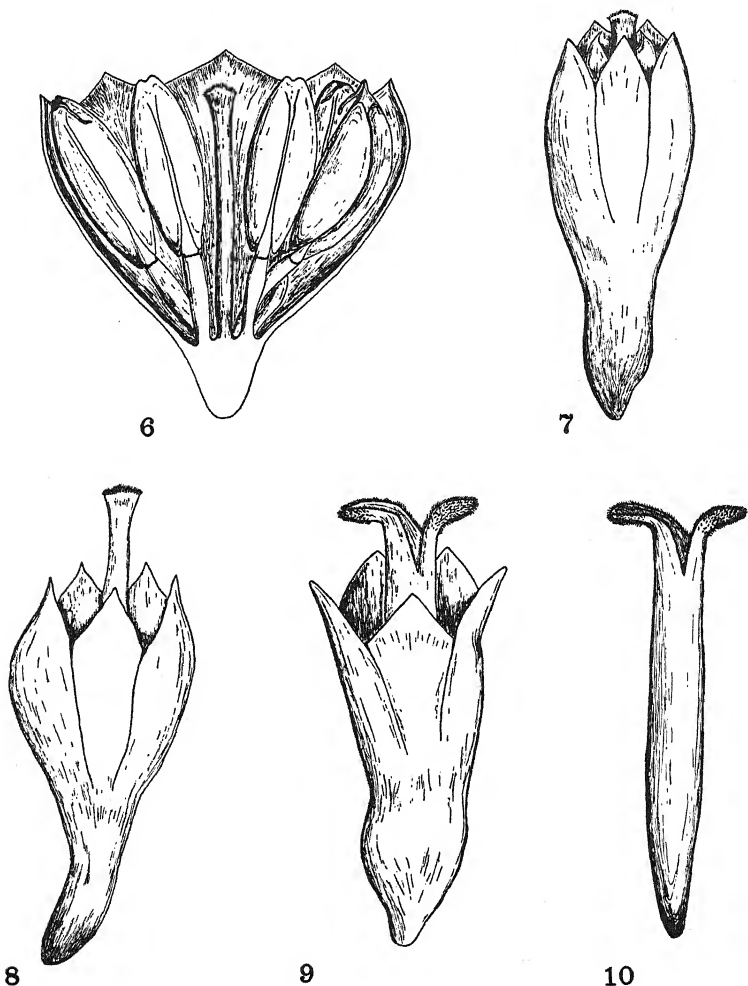


FIGS. 4, 5.—Fig. 4, pistillate head in longitudinal section; fig. 5, diseased pistillate head with two anthers in supernumerary bracts.

in which typical leaves have been observed in an inflorescence, and it is also the sole example of a form 1 plant producing several pistillate heads terminally. Over 10,000 plants of this form have been cultured.

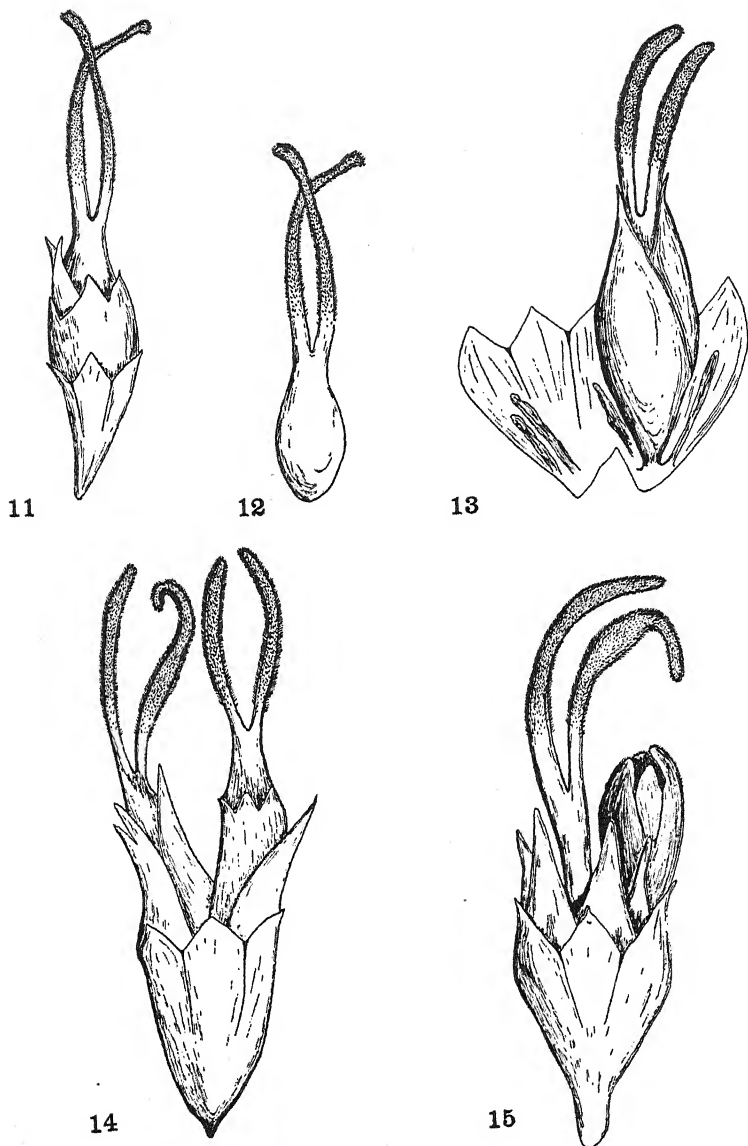
Late in the season, after seeds have set, a few plants of forms 1 and 2 have developed sterile pistillate heads from vestigial pistils of old staminate flowers. A staminate flower (fig. 6) has a perianth of one whorl which is funnelform and five-toothed; the five anthers are

distinct, and surround a vestigial pistil which is uniformly cylindrical except for a dilated tip. As the flower ages, the vestigial pistil



FIGS. 6-10.—Fig. 6, staminate flower in section; five stamens and vestigial pistil; fig. 7, staminate flower, pistillodium elongated; fig. 8, pistillodium further elongated; fig. 9, pistillodium bifurcated; fig. 10, pistillodium of fig. 9.

usually elongates so as to extend beyond the other floral parts (figs. 7, 8). In a few plants the dilated tip bifurcates and continues to elongate into a forked style with stigmatic surfaces, and the base of



FIGS. 11-15.—Fig. 11, pistillate head within staminate flower; two series of bracts, perianth of old staminate flower at base; fig. 12, pistil from fig. 11; fig. 13, staminate flower with perianth dissected to show four old stamens and pistillate head adhering to corolla; fig. 14, staminate flower in which two pistillate heads have formed; fig. 15, staminate flower in which pistillate head and staminate flower have formed. Edges of perianth of new staminate flower have stigmatic surfaces.

the pistillodium enlarges to resemble an ovary (figs. 9-12). As the pistillodium expands, it becomes ensheathed by an involucre of more or less separate bracts which develop adventitiously from the perianth at its base (figs. 11, 13). The pistils have not been found to



FIG. 16.—Androgynous raceme with pinnatifid leaves developed on plant of form 1 by increasing photoperiod after raceme bud appeared.

produce ovules; instead the ovary may be hollow, a solid parenchymatous structure, or it may even harbor an aborted staminate flower. Many variations occur. Figure 14, for example, shows two pistillate heads within the same staminate flower, and figure 15 shows a pistillate head and a staminate flower within the same original staminate flower. In the latter example the newly formed staminate flower possessed stigmatic surfaces along the edges of the corolla.

Several plants of forms 1 and 2 pollinated in May, because of an early planting, and these were continued in culture both out-of-doors and in the greenhouse in order to duplicate the late-season effect. Some plants were heavily fertilized, as SCHAFFNER (9) has shown that this often results in a reversal from the staminate to the pistillate condition. Although the plants lived through August, no change in floral development occurred, so that apparently only a very few plants, probably of a certain genotype, can be stimulated to develop pistillate heads within old staminate flowers. ROSA (8) reported a similar case in spinach in which the late-season conditions affected the floral type of only one strain.

The writer chanced to observe some sections of pistillate heads of form 1 which produced anthers containing normal appearing pollen within supernumerary bracts (figs. 4, 5). The young seed and certain other tissues of the head had degenerated, although no disease was noticed when the material was collected and no organism can be recognized in the tissues. Lateral pistillate heads elsewhere in the species have shown no staminate rudiments, so that it was surprising to find this modification in the most stable form.

### Discussion

The environment has a role in the floral development of *Ambrosia elatior* although this varies greatly with the strain. This has been noted frequently for other species; for example, in *Zea mays* (1, 7) and in spinach (8). The ratios of the floral types of cultures derived from monoecious form 2 and pistillate plants were readily altered by the environment, but the predominating type maintained its relative position so that only a few plants could have been affected. In these plants the inherited tendency to produce pistillate heads laterally was maintained, but the action of genes for floral expression in the racemes was changed in certain regions, or sometimes in an entire raceme. The individuals so affected must have had a gene complex somewhat different from their more stable relatives subjected to the same environment.

Monoecious form 1, on the other hand, developed true to type under all conditions, except where the photoperiod was increased after floral buds appeared. In this form the gene complex which

affects the development of staminate and pistillate flowers must be so differential that they formed in definite regions and at a particular time in the ontogeny in most environments. The change produced by disease and by a late season occurred in flowers which had formed and functioned normally. A floral part, like any other structure of a monoecious plant, has potentialities in genes for staminate and pistillate expression, even though one of these has been suppressed, because the time of action of genes is itself inherited. Every staminate flower of *Ambrosia elatior* has a vestigial pistil, which suggests its potential dimorphism, and the environment may accentuate this condition by causing the vestigial pistil to differentiate into a sterile pistillate head.

### Summary

1. This report is subsidiary to a genetical study on the floral types of *Ambrosia elatior* L., recently published.
2. The effect of the environment was determined by comparing the ratios of floral types in treated cultures with a control derived from seed of the same plant of known breeding behavior.
3. The influence of the environment in altering the ratios varied with the strain employed. The monoecious form which is widespread in nature was very stable, and was affected only by an increase in photoperiod after floral buds had appeared. Other forms had the ratios of their floral types considerably altered by removal of plumules, by growth under a photoperiod of 20 and 24 hours, and by waterlogged soil. However, the type which predominated in the control always remained dominant in treated cultures.
4. Old staminate flowers of a few plants of different forms developed non-functional pistillate heads as a late season effect.
5. Diseased pistillate heads in the leaf axils developed anthers in supernumerary bracts.

The writer is indebted to Professor H. H. BARTLETT for many helpful suggestions.

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# LEPIDOCARPON SPORANGIA FROM THE UPPER CARBONIFEROUS OF ILLINOIS

FREDDA D. REED

(WITH TEN FIGURES)

## Introduction

Reference to *Lepidocarpon* first appeared in 1877 (9), under the name of *Cardiocarpon anomalum*. WILLIAMSON applied the term to some isolated, mature, seed-like structures which in tangential section are in such close agreement with *Cardiocarpon* that CARRUTHERS (9) believed them to be identical with his *Cardiocarpon anomalum*, a genus of undoubted gymnospermous origin. At the time WILLIAMSON expressed some doubts as to the identity of the two specimens, doubts which were later justified by the investigations of SCOTT. In 1900 WILD and LOMAX (7) established the lycopodiaceous affinity of the cone of WILLIAMSON'S *Cardiocarpon*. Somewhat later SCOTT (6), working with the same and additional material, substantiated the discoveries of WILD and LOMAX, and further described a strobilus exhibiting differential development. This strobilus, bearing mature, integumented *Cardiocarpon*-like megasporangia at the base and immature lepidostroboïd megasporangia at the top, confirmed the relationship of the previously described isolated sporangia. The axis of this cone and the sporophylls were not to be distinguished from the axis and sporophylls of *Lepidostrobus*, but SCOTT believed that the fructification as a whole was "too distinct to be simply left in that genus," and so he established the generic term *Lepidocarpon* for its reception.

Isolated specimens of *Lepidocarpon* have been found in American coal ball material. KRICK (4) described *L. lomaxi* found in a coal ball from coal no. 5, Harrisburg, Illinois. These specimens were mature sporangia enveloped by the lamina-like processes which have their origin in the pedicel of the sporophyll; it is at this stage in the ontogeny of *Lepidocarpon* that sections, cut tangentially to the axis of the cone, bear a superficial resemblance to longitudinal sections of



integumented ovules of some primitive gymnosperms, for example, *Cardiocarpon*.

Other specimens have been found in coal no. 6, Petersburg, Indiana (5). They were immature sporangia detached from the strobilus before the delayed laminal development, and in so far as could be determined, their general topography and structure more nearly approximated the sporangia and sporophylls of *Lepidostrobus*. The possession of a single functioning megaspore, almost filling the sporangial cavity, established this latter form in the genus *Lepidocarpon*.

#### Material

The *Lepidocarpon* sporangia here described were found in coal ball no. 236 of the Harrisburg collection. The collection was made by Dr. A. C. NOÉ under the auspices of the Illinois State Geological Survey, from coal seam no. 5, Harrisburg, Illinois, which belongs in the Upper Pennsylvanian horizon. The sections are now a part of the paleobotanical collection, Department of Botany, University of Chicago.

The coal ball was a heterogeneous mass of plant structures imbedded in a matrix consisting largely of calcium carbonate and pyrite. In it were identified fragments of stems, roots, leaves, and sporangia of different species of ferns; stem tip, root, and leaf of *Sphenophyllum*; leaves and cortical tissue of *Lepidodendron*; petiole of *Medullosa*; and leaves of *Cordaitea* in addition to some sixty specimens of *Lepidocarpon* sporangia. These sporangia were detached from the axis of the strobilus and separated from one another. They varied in size as well as in age; but remembering the acropetal development and the wide variation in the size and in the stage of maturation of the sporangia contained in the strobilus described by SCOTT (6), one can regard these sporangia as having originated from the disintegration of a single cone.

#### Description

SPOROPHYLL.—The sporophyll is of the usual *Lepidostrobus* type, consisting of a radially extended part (radial with respect to the axis of the strobilus), referred to subsequently as the pedicel, and the up-turned leaf-like laminal portion. In its general topography it is con-

formable with that of *L. lomaxi* already mentioned and reproduced in figure 1. Figures 2, 3, and 4, from the Harrisburg material, are diagrams of separate sporangia but represent tangential sections from the proximal, middle, and distal regions of the pedicel or from the levels *a*—*a*, *b*—*b*, and *c*—*c* of figure 1. Figure 2, a somewhat oblique and crushed tangential section, shows the prominent dorsal

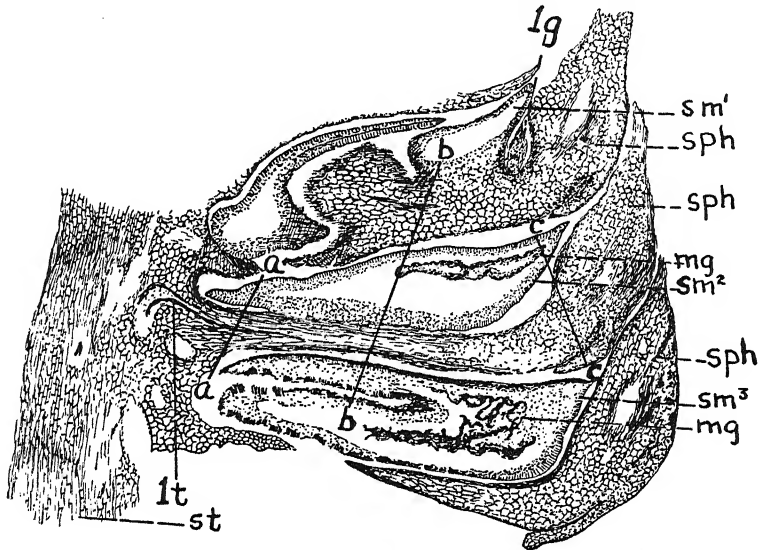
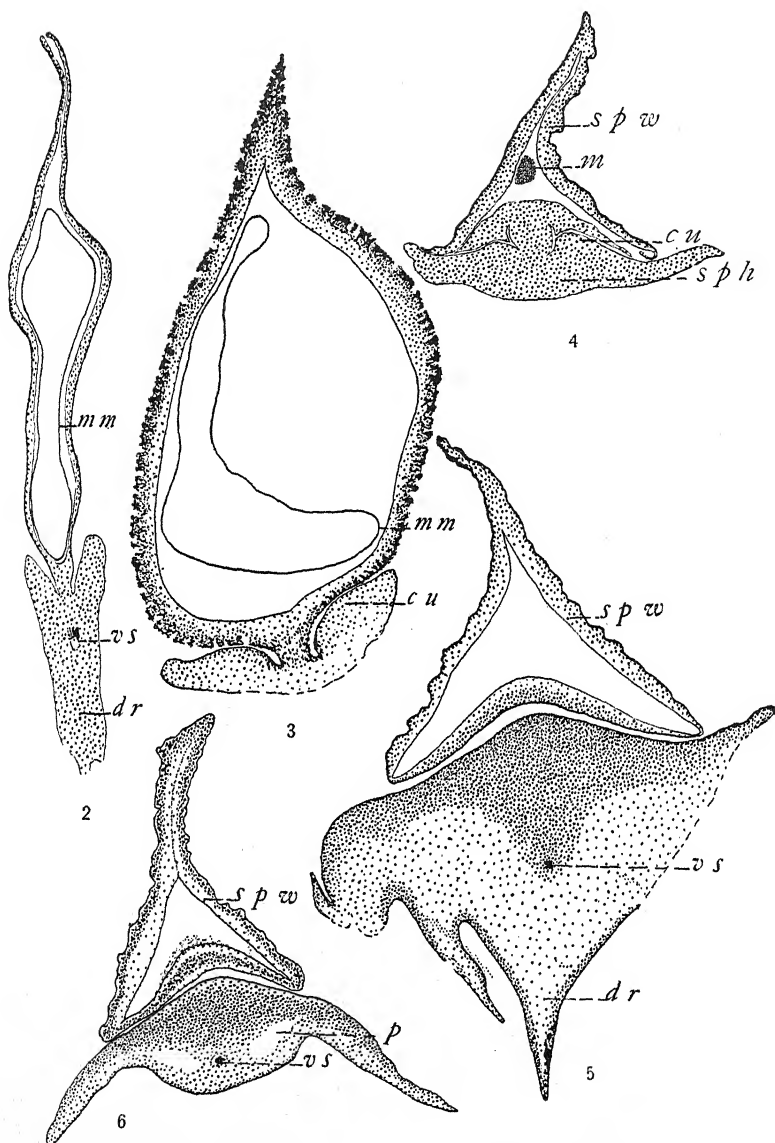


FIG. 1.—*Lepidocarpum lomaxi*, approximately radial section of strobilus: *st*, stele of axis in oblique section; *lt*, leaf trace bundle; *sph*, sporophylls, of which three are shown, each with horizontal pedicel and ascending lamina; *sm¹*, *sm²*, *sm³*, sporangia belonging to the three sporophylls; *lg*, ligule; *mg*, remains of megaspore.  $\times 21$ . After SCOTT (6).

rib; at the proximal end of the pedicel it forms a keel-like projection which becomes confluent with the axis of the cone. For most of its length the pedicel is much thinner vertically, but the margins are expanded into lateral cushions (*cu*, figs. 3, 4) which partially surround and presumably support the base of the sporangium. Toward its distal end the pedicel becomes massive, in transverse section appearing as a broad triangle with lateral wings (fig. 5); at this level the sporophyll not only invests its sporangium but the abaxial surface fits neatly over the sporangium below. From this broad triangular zone the sporophyll bends at right angles, forming the



FIGS. 2-6.—*Lepidocarpon*. Fig. 2, oblique tangential section of sporophyll and sporangium from proximal region: *dr*, dorsal rib; *vs*, vascular strand; *mm*, megaspore membrane.  $\times 14$ . Fig. 3, tangential section of sporophyll and sporangium through middle region: *cu*, lateral cushions of sporophyll.  $\times 12.5$ . Figs. 4-6, tangential sections through distal extremities of sporangia and sporophylls: *sph*, sporophyll; *sphw*, sporangial wall.  $\times 14$ .

laminal extension which covers the end of the sporangium and overlaps the sporophylls above. Thus the pattern of the sporophyll is so designed that the assemblage would indicate a relatively tight and compact cone with closely enveloped sporangia.

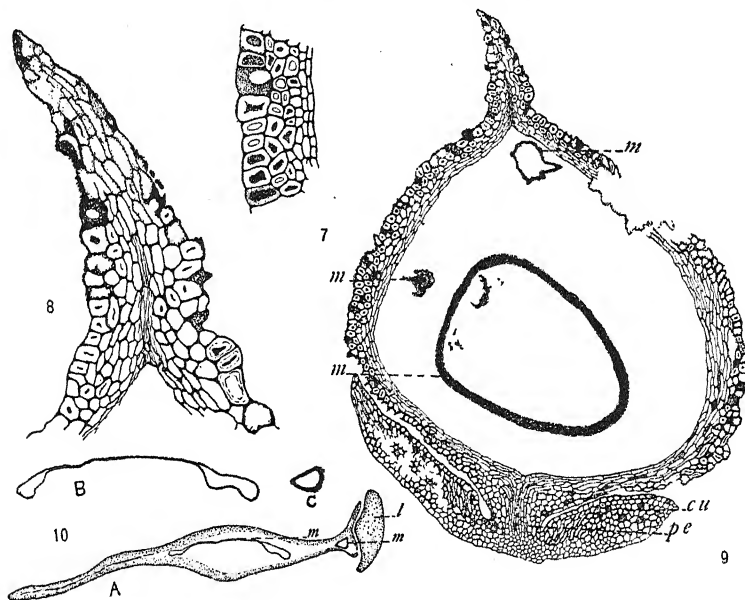
A single vascular strand traverses the length of the sporophyll; in this material it is so poorly preserved, however, that there is no remaining detail of structure, only the course of the strand being indicated. Figure 6, a transverse section of the lamina, shows two lacunar areas occupying the position of parichnos of vegetative leaves of *Lepidodendron*; and since SCOTT, working with more perfectly preserved material, found identical structures in the lamina and vegetative leaves, there seems no reason for doubting the parichnos origin of these areas. In *L. lomaxi* (fig. 1) there is a ligule situated on the adaxial surface of the sporophyll between the sporangium and the lamina. Failure to find any trace of a ligule here is not to be attributed to its absence in the living material, but rather to lack of preservation.

**SPORANGIUM.**—Located on the adaxial surface of the pedicel and fitting into the groove made by the lateral cushions is the sporangium. Its attachment is a very narrow one tangentially (figs. 3, 4, 9), but radially it extends almost the length of the horizontal portion of the pedicel. The sporangium is elongated radially and deep vertically; it is broad at the base but narrows sharply toward the top, resulting in a sharp ridge which gives the tangential section the appearance of a sporangium with a pointed apex. At the proximal end the sporangium is round at the base, toward the distal end it follows the curvature made by the marginal wings of the sporophyll, and at this level it is triangular in tangential section with the two basal angles, as well as the apical angle, sharply acute (figs. 4-6).

The sporangial wall, which at the basal end merges imperceptibly with the tissues of the pedicel, is of two layers (figs. 7, 8), each averaging about three cells thick. The epidermis is scarcely to be distinguished from the hypodermis, both being composed of thick walled, roughly isodiametric cells, except perhaps for the slightly thicker walls and the larger size of the cells. It does not have the distinct palisade appearance of *L. lomaxi*, or as described for *Lepidostrobus* (2). The cells of the inner layer are thin walled and elongated

in the plane of the surface of the sporangium (fig. 9). There is no trace of a tapetal layer, nor is there any indication of a sub-archesporial pad; on the contrary the more nearly median sections show a slight furrowing at the base of the sporangial cavity (figs. 3, 9).

MEGASPORE.—In the younger sporangia vestiges of all four spores are found, three of which are shown in figure 9, and their position



FIGS. 7-10.—*Lepidocarpon*. Figs. 7, 8, detail of portion of sporangial wall.  $\times 43$ . Fig. 9, tangential section of sporophyll and sporangium showing functioning megaspore and two of three aborting ones: *pe*, pedicel of sporangium, semidiagrammatic.  $\times 20$ . Fig. 10 (A), section of sporophyll and sporangium transverse to axis of parent cone: *l*, lamina.  $\times 14$ . (B, C), functioning and aborting megaspores from A showing difference in thickness of megaspore membranes.  $\times 55$ .

with relation to one another suggests their origin from the tetrahedral type of division of the megaspore mother cell as usually occurs in pteridophytes. The size of the three aborting megaspores and the thickness of their membranes show clearly that they did not disintegrate immediately after inception, but that there was a period of growth and development of all four megaspores alike during their early history. Soon, however, the proximal spore gained ascendancy

over the other three (fig. 9) and increased enormously in size, at length filling the sporangial cavity (fig. 3). Meanwhile the sporangium had increased in size so that the largest of the spores (fig. 3) are more than twice the diameter of the one shown in figure 9. With increase in diameter of the functioning spores there is a corresponding decrease in the thickness of the megaspore membrane; that of the megaspore of figure 9 is at least  $50\ \mu$  thick, whereas the membrane shown in figure 10 measures about  $18\ \mu$ . Again, figure 10 shows the variation in thickness of the membranes of the functional megaspore and one of the aborting megaspores of the same sporangium. In the largest of the sporangia the megaspore membrane is usually collapsed (fig. 3), or it may appear broken and fragmented, again it may have disappeared altogether; only rarely is it ever seen intact and certainly never did I find any tissue that could be interpreted as gametophyte. There are fragments of tissue occasionally met with inside the membrane, but it is clearly intruded material.

### Discussion

It has been stated of *Selaginella* (3), "The retention of the spores within the sporangium during gametophyte development, fertilization, and embryo formation suggests seed structure, especially where but one spore of the tetrad develops." Again (1), "In extreme cases there is little dehiscence of the sporangium and the megaspore remains inside, so that the shoot with its cotyledons and stem tip, and the root, break through the sporangium. In this extreme case the term "seed" is strictly applicable to *Selaginella*." True, in such cases, the megaspore has been retained and under such conditions the term seed might seem to be applicable. But in *Selaginella* the sporangial wall is so constituted that it could be of little or no further value in the nutrition of the gametophyte. The developmental story of the megaspore membrane is one of increasing complexity and thickness, with consequent increase in the difficulty of food absorption from the surrounding tissues. Furthermore, the volume of the sporangium is much greater than that of the functioning spore or spores, so that they are free in a big cavity, which would likewise increase the difficulty of food assimilation from the sporangium even though it were available. Moreover, in instances when only one of

the four megaspores becomes functional, its selection appears to be wholly fortuitous and not determined by any particular advantage of position within the sporangium. Consequently, the megaspores of *Selaginella* are of necessity wholly independent of the parent plant for their further development (that is, formation of the archegonium, fertilization, and evolution of the embryo), and any cases of their retention must be attributed to some external ecological factor which would prevent the dehiscence and collapse of the sporangial wall with the resulting liberation of the spores. Under such circumstances there is no physiological relationship existing between the spore and the sporangium and its retention is only apparent, not real.

On the other hand, while these specimens of *Lepidocarpon* were on the whole not so well preserved as those from the English Coal Measures, yet in the preceding description there are a few salient features of these young "un-integumented" sporangia that demonstrate the advance made by Paleozoic pteridophytes, and indicate the stages, beyond the acquisition of heterospory, through which spermatophytes must have passed in attaining the seed habit. They are: (1) the relatively massive sporangial wall, differentiated into an outer protective layer and an inner nutritive layer; (2) the early selection of the chalazal megaspore for the functioning megaspore; (3) the position of the megaspore membrane in contact with the sporangial wall, making possible the assimilation of food; and (4) the decrease in the thickness of the megaspore membrane, thereby facilitating food assimilation. Thus, although we have as yet no material evidence of the retention of the megaspore of *Lepidocarpon*, yet possession of these characters would result in the production of a dependent gametophyte surrounded by living and functional tissue and physiologically retained within it.

### Summary

1. Some young "un-integumented" specimens of *Lepidocarpon* from the Harrisburg, Illinois, coal ball collection are described. The geological horizon of the material is Upper Pennsylvanian.
2. The sporophylls consist of a radially extended stalk which at the proximal end is decurrent on the axis of the strobilus, and dis-

tally broadens into the leaf-like laminal portion. The lamina, extending at right angles to the stalk, covers the end of its sporangium and overlaps the sporophylls above. The sporophyll is traversed by a single vascular strand.

3. The sporangia, borne singly on the adaxial surface of the stalk of the sporophyll, are distinguished by the possession of a massive wall differentiated into two general layers, an outer protective layer and an inner nutritive layer.

4. Evidences were found of four megaspores in a sporangium, three of which aborted. The differential development displayed by the various specimens indicated an early selection of the chalazal megaspore as the functioning megaspore. With increase in size of the functioning megaspore there was a progressive decrease in the thickness of the megaspore membrane.

5. From an analogy of developing sporangia and megaspores of *Selaginella* and *Lepidocarpon*, the following conclusions are derived: any case of a megaspore of *Selaginella* remaining within the sporangium is due to some chance external factor and hence is no nearer the seed habit than the case of a megaspore that is shed; whereas, to *Lepidocarpon*, a sporangium of living and functional tissue containing a dependent gametophyte, the term "seed" seems "strictly applicable."

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# DEVELOPMENT OF THE MEGAGAMETOPHYTE AND EMBRYO OF ALLIUM MUTABILE<sup>1</sup>

THOMAS REGINALD PORTER

(WITH TWENTY-SEVEN FIGURES)

## Introduction

This work was begun with the idea of comparing the developmental morphology of the wild onions (*Allium canadense* L. and *A. mutabile* Michx.) growing around Lincoln, Nebraska, with that of *Allium cepa* L. described by HOFFMAN (3). The development of the megagametophyte of one of them proved so interesting that the emphasis was placed there. Apparently these species of *Allium* have not been previously investigated.

At first both *Allium canadense* and *A. mutabile* were utilized, but the former was abandoned because it produced bulblets and only a few flowers. The latter always produced seeds in abundance.

Material was collected in 1934 and 1935; that collected in the late spring of 1934 proved unsatisfactory because of continuous drought. The material collected during the season of 1935 was reasonably satisfactory although it endured drought late in the season. The abbreviation of the development of the megagametophyte described later might be due to weather conditions, but it appeared to be the usual one.

During a period extending from May to July in 1935, flower buds in all stages were collected and fixed. Formal-acetic-alcohol of the following formula was employed: neutral formalin, 10 cc.; glacial acetic acid, 5 cc.; 50% alcohol, 100 cc. Because of the drought, care was taken to effect complete hydration of tissues before fixation. All material was imbedded in paraffin with the exception of some seeds and embryos. These were cleared in cedar oil and observed thus.

Young flower buds and ovules were sectioned 8-12  $\mu$  thick; more mature ovules and the embryos were cut 3-5  $\mu$  thick. The greatest

<sup>1</sup> Contribution no. 100 from the Department of Botany, University of Nebraska.

difficulty in sectioning came when the older ovules were sectioned. This difficulty was overcome somewhat by prolonged periods in xylol and xylol-paraffin solutions.

### Investigation

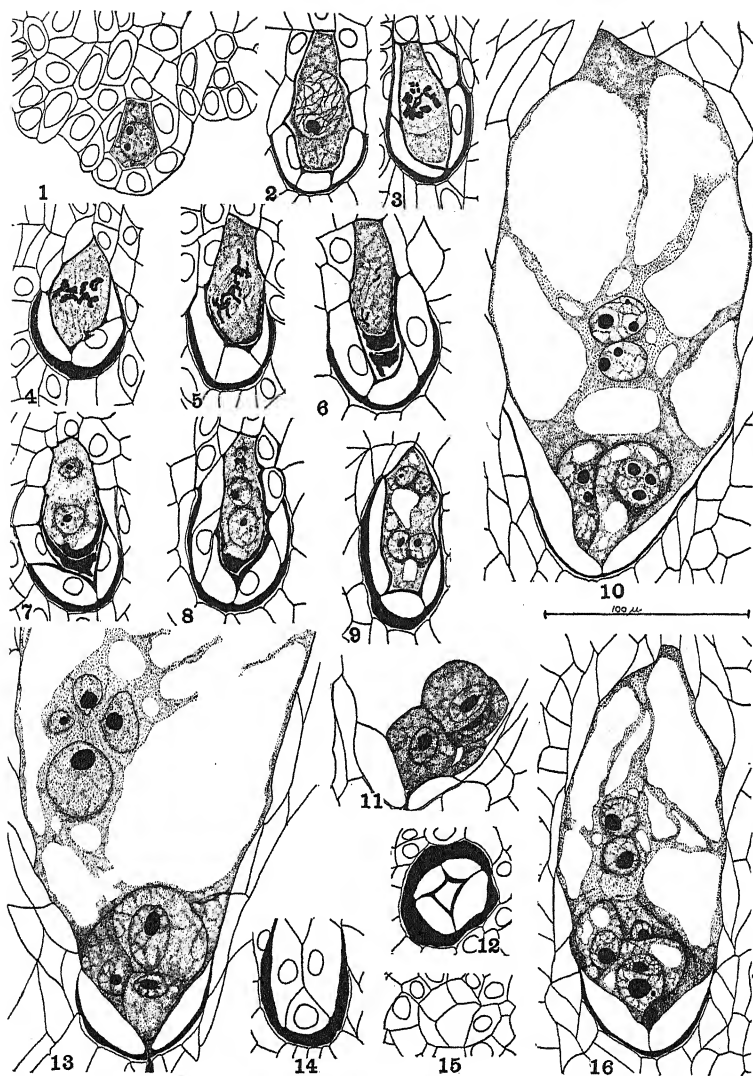
Pollination experiments showed that in all cases seeds matured whether the flowers were cross or self pollinated. The most efficient method of controlling pollination was by tying squares of cellophane over the flower heads, making a pouch with ample space. Superfluous flowers were removed. Material from these experimental heads was fixed every six or twelve hours for a period of six days; then every day for a period of several weeks.

OVULE.—The ovule is essentially of the *Lilium* type. It arises at the base of the ovary, is anatropous, and the funiculus is short. At the time the archesporium becomes evident the rudiments of the inner integument (fig. 1) are fairly well formed and the outer integument is beginning to develop. Both integuments have enveloped the ovule completely before meiosis. The inner integument is from two to four cells thick while the outer attains a thickness of nine cells. At the micropyle the nucellar tissue is separated from the integuments by a distance of about 2–3  $\mu$ . At maturity the inner integuments are shorter than the outer. Both are tightly pressed together. At the time of pollination the micropyle dilates. Immediately after the entrance of the pollen tube the integuments again become pressed together.

At maturity, the micropylar and chalazal ends of the megagametophyte are parallel to the funiculus. After fertilization, as the embryo develops, the megagametophyte becomes horseshoe-shaped and widens at the chalazal end to about three times the width of the micropylar end. Later the chalazal end becomes bifurcated by ingrowing sporophytic tissue.

Of the six ovules forming in the young pistil, usually only two or three reach maturity. Ordinarily abortions start about the time that fertilization would normally take place. In some cases as many as six ovules mature.

NUCELLUS.—The nucellus is unusual in that the cells at the extreme micropylar end are larger, thicker walled, and differently



FIGS. 1-16.—Fig. 1, young ovule showing archesporium; figs. 2-4, archesporium undergoing meiosis, outer nucellar walls thickened; figs. 5-7, first division of functional megaspore, one disintegrating; fig. 8, second division in megagametophyte; spindle apparent between small antipodal nuclei; fig. 9, four megagametophyte nuclei before orientation and after disintegration of antipodals; fig. 10, mature megagametophyte; fig. 11, three-celled embryo, pollen tube persisting at right; fig. 12, cross section of nucellar cells at micropyle; fig. 13, two celled embryo, synergid at left, empty pollen tube at right, endosperm cells above; fig. 14, longitudinal view of nucellar cells at micropyle; fig. 15, end view of nucellar cells at micropyle; fig. 16, fertilization, sperm nucleus pressed against egg, synergid on left of egg, pollen tube with second male nucleus on right of egg, disintegrating tube nucleus near micropyle.

shaped from those of the rest of the nucellus. There are only four which differ greatly from the rest. Two of these are long cells which are blunt at one end and taper to the other (figs. 14, 15). The other two are equally large but pointed at both ends, and fit between the two cells having blunt ends (fig. 14). In cross section they are all of similar diameter (fig. 12). These cells are forced apart as the pollen tube enters, and after its protoplasm has moved into the megagametophyte they again come together (fig. 16).

The outer walls of these large nucellar cells are becoming thickened at the time of archesporial differentiation. These cell walls, as well as those of the sporophytic cells in contact with the rest of the megagametophyte (fig. 2), continue to thicken. They reach their greatest thickness at the time the nuclei of the megagametophyte are becoming oriented (fig. 9). The thickening gives the appearance of gelatinous layers. The walls in contact with the megagametophyte remain thickened until reduced by its enlargement as it approaches maturity. This decrease in thickness of the cell walls may be due in part to the absorption of some of the material by the enlarging female nuclei, as suggested by COULTER and CHAMBERLAIN (1). In these illustrations the heavy nucellar walls are shown with a single, heavy black line.

**MEGAGAMETOPHYTE.**—The archesporial cell is differentiated about three or four days before the flower bud opens (figs. 1, 2). It arises from a hypodermal cell and differs from the rest of the cells in density of protoplasm and in size of cell and nucleus. No case was found in which more than one mother cell occurred. No primary parietal cell is formed, the first division being meiotic (figs. 3-5) and resulting in two cells separated by a wall. The one nearest the micropyle immediately disintegrates; the other undergoes one more division, thus forming two megaspores separated by a wall. The one near the micropylar end disintegrates at once (figs. 6, 7).

The remaining functional megaspore undergoes an unequal division, resulting in a large nucleus at the micropylar end and a smaller one at the chalazal end (fig. 7). After this division the megagametophyte enlarges, thus forcing the disintegrating megaspore toward the micropylar end and leaving it in finger-like strands along the wall of the gametophyte (figs. 6-8).

The division of the two nuclei already formed results in two small, ephemeral nuclei at the chalazal end and two larger, unequal ones at the micropylar end (fig. 8). The larger nucleus of these two is nearer the micropyle. Each of these two functional nuclei divides once more. The innermost produces two daughter nuclei of equal size which function as the two polar nuclei. The micropylar one produces two nuclei, one of which is slightly larger than the other (fig. 9). This larger nucleus becomes the egg; its sister nucleus, the long-lived synergid. By vacuolization of the cytoplasm these four nuclei become oriented as in figure 9. The disintegrating megaspore now becomes absorbed completely. Possibly its substance nourishes the megagametophyte. This stage was found most commonly in material collected a few hours before the flower opened. Between this stage and the one at which the megagametophyte is matured, these four nuclei become very different in appearance. The two polar nuclei are pale bodies with little chromatic material; the egg and synergid are of about the same density but can be distinguished by differences in size and vacuolization. They are separated from the surrounding protoplasm by membranes. The egg cell has a large vacuole between its nucleus and the micropyle, whereas the synergid is vacuolated only at the end farthest from the micropyle. The egg lies nearer the periphery of the megagametophyte than does the synergid (fig. 10).

When the megagametophyte has enlarged about fifteen diameters after its last nuclear division, the gamete is ready for fertilization. Now the megagametophyte is more or less elliptical, but slightly narrower and more pointed at the extreme chalazal end. By growth of the rest of the gametophyte this antipodal region at the chalazal end becomes a small pocket pressed into the surrounding tissue (figs. 10, 16). Because of further vacuolization in the cytoplasm of the gametophyte it enlarges still more. Most of the cytoplasm is massed around the egg and synergid, where it is also the most dense. A smaller amount is gathered about the polar nuclei and a still smaller amount lies at the chalazal end. The masses of cytoplasm are connected with each other and the peripheral cytoplasm by long, irregular strands. The megagametophyte was found to have reached maturity about six hours before the first anther opened, or about six hours after the flower had opened.

It was found from observing many megagametophytes that they range in size from 175 to 300  $\mu$  in length, and 78–123  $\mu$  in width. The larger gametophytes were found in pistils in which the greatest number of ovules had aborted.

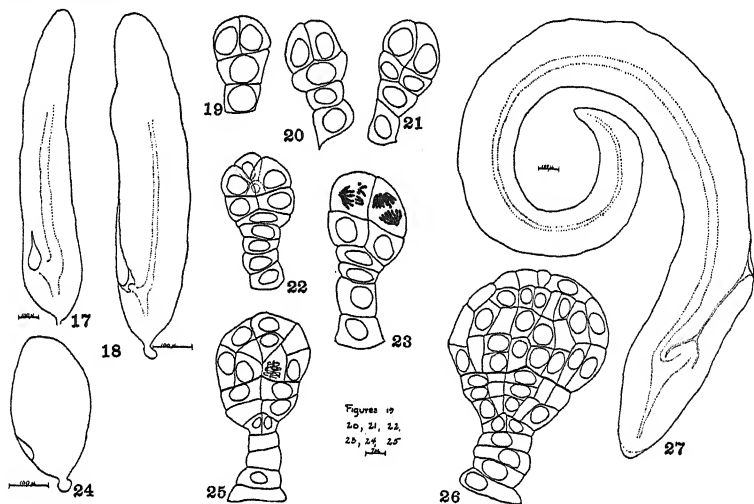
FERTILIZATION.—Fertilization occurs between twelve and twenty-four hours after the flower opens. From the papillate nutritive cells at the base of the ovule the pollen tube passes directly into the micropyle. The tube passes through it, forces apart the large nucellar cells at the micropylar end of the megagametophyte and enters on the side opposite the synergid, against which it presses. The first male nucleus passes into the cytoplasm of the egg and becomes tightly pressed against the egg nucleus (fig. 16). In this figure the second male nucleus may be seen in the pollen tube on its way to fuse with the two polar nuclei. The tube nucleus which is starting to disintegrate may be seen as a black spot between the nucellus and the egg. The pollen tube persists for some time, usually until after the embryo has reached the three celled stage (fig. 11).

At the time fertilization occurs the synergid is rich in protoplasm. It persists in this condition until the embryo has reached the two or three celled stage (fig. 13). This is between forty-eight and seventy-two hours after fertilization.

The polar nuclei become closely applied to each other by the time the second male nucleus has reached them. After triple fusion the endosperm nuclei divide rapidly for a time before the zygote divides. There is an unequal division here which produces endosperm nuclei of slightly different sizes. Figure 13 shows one complete endosperm nucleus and parts of three others. The spindle is still apparent between the new nuclei and they seem to be pulled to a point on the side toward the spindle. These nuclei soon migrate to the periphery of the sac, where they continue to divide. At maturity they are evenly distributed through the sac and about the embryo. Finally they become the nuclei of the usual cellular endosperm.

After fertilization, the outer integuments close together again as do also the nucellar cells at the micropylar end, although the latter are slower in closing. Immediately the megagametophyte begins to elongate at the chalazal end, doubles back toward the micropyle, and becomes horseshoe-shaped.

EMBRYO.—The first division of the zygote takes place about forty-eight hours after fertilization, and is transverse and unequal (fig. 13). The cell toward the micropyle is smaller and often irregular in shape, being pushed in on one side by the pollen tube and on the other by the synergid. The terminal cell is usually about twice as large as its sister cell. The latter will divide to form the suspensor and part of the embryo.



FIGS. 17-27.—Figs. 17, 18, 24, 27, successive stages in development of embryo; figs. 19-23, 25, 26, detail of early stages in development of embryo.

The second division in the terminal cell (fig. 11) is at right angles to the plane of the first division. This occurs sixty to seventy-two hours after fertilization. Usually these cells are oval in shape, with nuclei longer than they are wide.

The second division of the suspensor initial (fig. 19) forms a two-celled suspensor. The next division occurs in the cell adjoining the embryo and contributes one cell to the base of the embryo (fig. 20). This cell continues to divide to form the radicle and its parts. The two terminal cells of the embryo divide to form the cotyledon and stem meristem. The region in which the notch will develop is between the terminal cell and the one cut off from the suspensor (figs. 21, 22, and following). At maturity the suspensor is several cells long and two cells wide and thick (figs. 18, 24).



From this stage on it is hard to determine the sequence of the divisions since they take place rapidly. Figure 23 shows the mitotic division which is the beginning of differentiation of the primary histogens. At the stage shown in figure 25 the place where the notch will form in the embryo is becoming apparent (on the right side). At this stage the dermatogen has become differentiated over the cotyledon. Just above the fourth suspensor cell are the three layers of cells which develop into the radicle and the region producing the stem tip.

Figure 26 shows the enlarged embryo and further differentiation of the tissues. The dermatogen is evident extending around the embryo. Directly beneath the dermatogen is the periblem which is about two cells wide in the upper part and one in the lower. In the central portion of the embryo are the cells which form the plerome. The upper part of the embryo develops with great rapidity to form the long, coiled cotyledon; whereas the lower part, from the notch down, is somewhat slow in growth. Figure 24 shows the embryo advanced to a further stage. This surface view shows the notch well developed. Figure 18 shows the embryo in a still more advanced stage. The notch is nearly complete. The meristematic stem tip is now pushing up into the notch. The dotted lines indicate where the procambial strand has started to develop. This figure, as well as figures 17, 24, and 27, was drawn from entire embryos which had been cleared in cedar oil. Figure 17 shows the embryo further developed. The procambium, which appears as a dark band through the cleared tissue, is extending well toward the cotyledonary tip. The tip of the cotyledon is starting to curve backward. In figure 27 the mature embryo is shown.

The mature embryo lies curled around in the seed. It is completely surrounded by hard, starchy endosperm and in all respects is much like the one described for *Allium cepa* by HOFFMAN (3). The procambium lies in the same general position in the embryo, but that of the plumule is found to extend farther toward its tip than it does in *A. cepa*. It extends to the tip of the cotyledon, suggesting that the cotyledonary tip may be haustorial in nature. In this embryo the stem tip is more closely surrounded by tissue than is the one described by HOFFMAN. Furthermore, the notch is much deeper in this

species and is in the form of a narrow tube, widening at both ends (fig. 27). It does not have the characteristic tip described for the other species. The embryo of this species needs further investigation.

### Discussion

A number of variations have been reported in the development of the megaspores of monocotyledons (1, 4), but relatively few cases have been reported where but two megaspores were produced, one disintegrating and one producing the nuclei of the megagametophyte. *Allium mutabile* is like *Chlorophytum orchitastrum* (5) so far as the megaspores are concerned. Its type of megaspore disintegration is that of type C of RUTGER's second scheme, as given by SCHNARF (4). The one megaspore disintegrates as he shows, but unlike the one reported by SCHNARF, the first two nuclei in the megagametophyte are unequal. STRASBURGER (7) and SCHURHOFF (5) report two megaspores developing in *A. fistulosum* and *A. odorum*, the micropylar one disintegrating and the lower one dividing to form the megagametophyte. The variation between these and *A. mutabile* is not great. According to COULTER and CHAMBERLAIN, this would seem to indicate that the former two plants are as high in the plant kingdom as *A. mutabile*.

At maturity the megagametophyte contains only the essential cells. This condition would seem advantageous since this plant has a relatively short growing season. While four nucleate megagametophytes have been reported for *Clintonia* by SMITH (6), for *Cypripedium* by PACE (8), and for *Plumbagella* by DAHLGREN (2), the condition in *Allium mutabile* is actually different.

STRASBURGER (7) reports a megagametophyte for *Allium fistulosum* consisting of eight or nine nuclei, a large egg, unequal synergids, two polars, and several antipodals.

After the entrance of the pollen tube, the egg apparatus of *A. mutabile* superficially resembles that pictured by STRASBURGER, in that the large pollen tube lies in the position occupied by a synergid in his species. In this study the pollen tube was thus mistaken for a time.

The mature embryo compares essentially with that of *Allium cepa* (3), except for minor exceptions already mentioned. The notch develops in about the same region as reported by SCHNARF for *Luzula forsteri*. It develops lower on the embryo than he reports for *Poa annua*.

The thick walled nucellus is apparently uncommon; at least it has not been reported in accounts of other species of *Allium*. This condition probably accounts for the persistence of the nucellar cells, the thick cell walls being digested to provide food for the growing female nuclei. This variation, however, might be due to the conditions of drought which prevailed at the time the study was made.

### Summary

1. In *Allium mutabile* two megaspores are produced; of these one disintegrates, while the other forms the megagametophyte.
2. The first division of the functional megaspore results in a large nucleus at the micropylar end and a smaller one at the antipodal end.
3. The larger nucleus divides twice to form the egg, one synergid, and two polar nuclei of the megagametophyte.
4. The smaller (antipodal) nucleus divides once to form two ephemeral antipodal nuclei.
5. At maturity the megagametophyte consists of a large vacuolated egg, a smaller synergid, and two still smaller polar nuclei.
6. The embryo develops normally and closely compares with those reported for other species of *Allium*.
7. Results of hand pollination show that both self and cross pollination are effective.
8. The cells of the nucellus are unusually heavy walled.

The writer wishes to express his appreciation to DR. ELDA R. WALKER for directing this study, and to others for their assistance.

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# A FOSSIL ARAUCARIAN EMBRYO FROM THE CERRO CUADRADO OF PATAGONIA

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 476

BERTHA SCHWEITZER DARROW

(WITH THIRTEEN FIGURES)

## Introduction

Although much fossil wood identified as Araucarian has been described, only a limited number of complete cones and isolated cone scales have been found. Dr. ELMER RIGGS of the Field Museum Patagonian Expedition (1924) discovered an extensive fossil forest at Cerro Cuadrado, Patagonia (13). More than one hundred excellently preserved cones were collected and these have made possible the study and description of the fossil embryo of at least one species. Dr. G. R. WIELAND has examined this material (13) and named the species *Proaraucaria mirabilis*. They have been referred both to the Triassic (13) and to the Lower Eocene (3).

The cones of two other species, *Araucarites windhausenii* (4) and *A. mirabilis* (12), were also found in Patagonia, in close proximity to the Cerro Cuadrado forest where Dr. RIGGS collected. Both of these species are regarded as identical with *Proaraucaria mirabilis* (Speg.) Wieland, but from the illustrations in the papers just mentioned, it seems that the Field Museum cones are in a far better state of preservation than any of those figured by either GOTHAN or SPEGAZZINI. The embryo is not referred to by GOTHAN, and SPEGAZZINI states that the *Araucarites mirabilis* cones were immature, embryos having not as yet developed when the cones were subjected to silicification.

With the exception of some foliage material of *Araucarites nathorstii* Dus. (2) from Punta Arenas on the Straits of Magellan, and two fragmentary cone scales of *Araucarites patagonica* (7) and *Araucaria* sp. (1), the fossils of the Cerro Cuadrado petrified forest (including the *Araucarites mirabilis* Speg. and the *A. windhausenii* Goth.) represent the only Araucarian fossils thus far described from South America.

SEWARD and FORD (10) discussed both the living and fossil Araucarians, and SEWARD (9) has reviewed all of the cone and foliage material described before 1919. GOTHAN (5) has since described a cone, *Conites araucarioides*, from the Tendagura of Africa, and another, *Araucarites obscurum* Wiel. (13), has been described from Como Bluff, Wyoming.

### Methods

A preliminary study was made of all the *Proaraucaria mirabilis* cones in the Field Museum collection, especially those which Dr. WIELAND had cut and polished, in order to select for subsequent sectioning those showing exceptional preservation. Cone 13854 (Field Museum number) was finally chosen for detailed study because of its large size and because a tangential cut previously made by Dr. WIELAND revealed the presence of embryos.

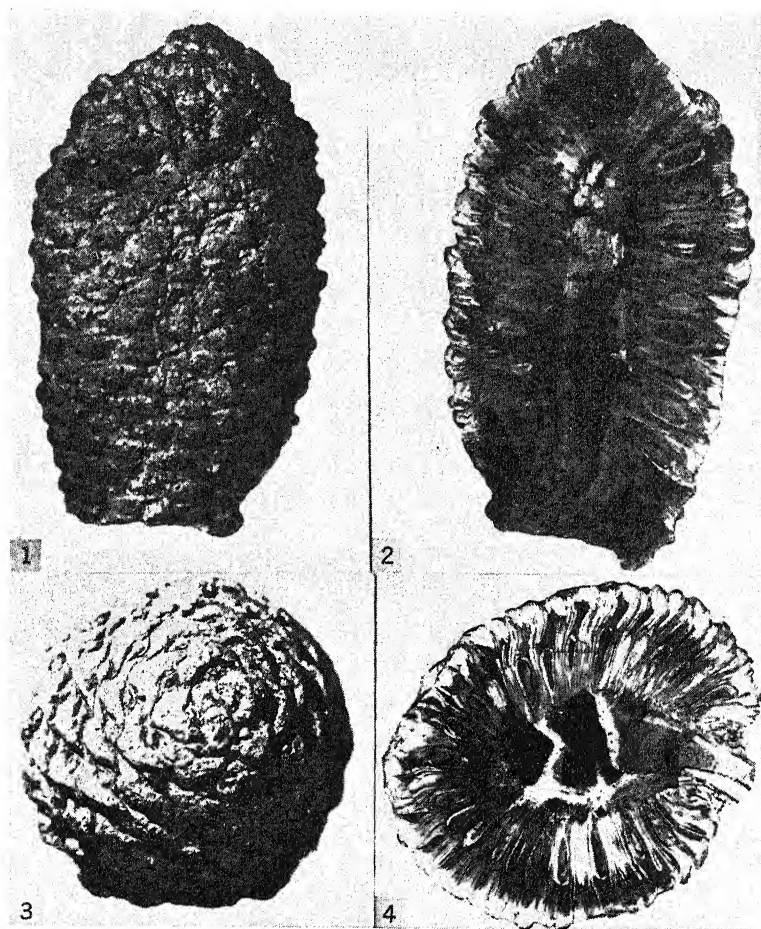
Ordinary methods for making petrological sections were used. Some thin sections were made, but in most cases polished slabs showed more detail than sections. A continuous series of transverse and longitudinal sections of the embryo of *Araucaria araucana* Koch., which grows at present in the Andes of Chile, not far from the locality of the fossil forest, was studied for comparison with the fossil specimens.

### Description and discussion

CONE AND CONE SCALES.—Although one of the largest cones of the series of *Proaraucaria mirabilis*, number 13854 is small when compared with those of modern Araucarians, certain species of which have carpellate cones exceeding 30 cm. in length. The fossil cone (figs. 1, 2) measures 88 mm. in length and 50 mm. in diameter. The exterior is reddish brown. No tips of the cone scales were found. These may never have been present since other cones which show no erosion do not have a spine at the tip of the scale as do living species. The fossil may have been like the living *Araucaria angustifolia*, in which the spine falls off when the cone becomes mature. The ligule is conspicuous (fig. 3).

Internally the cone is composed of a jaspersy silica of uniform, non-porous texture. The colors range from scattered spots of bril-

liant red, through black-brown, yellow, buff, milky blue, and white. Portions are colorless and transparent. There seems to be a marked



FIGS. 1-4.—Fig. 1, external view of half of *Proaraucaria mirabilis*, cone 13854; fig. 2, median longitudinal section of cone 13854; fig. 3, view looking down on apex of *P. mirabilis*, cone 13837; fig. 4, median longitudinal section of cone 13821; internal structure particularly well preserved.

tendency in this cone, as well as in others, for the various tissues of the seed and cone scale to have become impregnated in petrification with specific colors of quartz, almost as if the difference in tex-

ture of the living determined the color of the fossilized tissues. For instance, the embryo is generally a light buff color, but in exceptional cases is a dark smoky, semi-opalescent brown. The vascular strands in the buff embryos are a light brown, so that they appear in good contrast to the general ground color. The megagametophyte is milky white, while the lumen formerly occupied by nucellar tissue is transparent. The remaining fragmentary nucellar tissue is milky white. The integument is always a light buff, and the other tissues of the cone scale are usually dark, but may be yellow, white, or a mixture of colors.

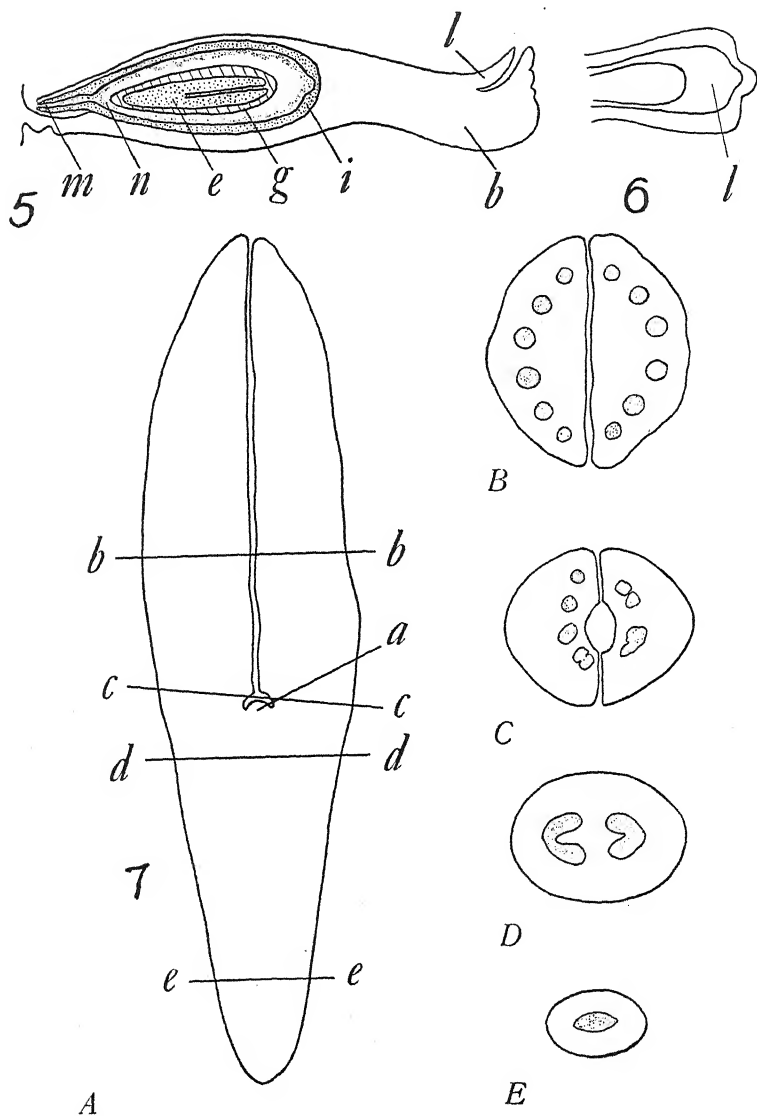
Cone 13821 of the Field Museum collection, being in general lighter in color, shows to better advantage this specific color distribution (fig. 4).

The pith of cone 13854 measures 10 mm. in diameter, and although it is smaller in this cone than in others of the collection, it is relatively large, as in modern Araucarian cones. It is bordered by a delicate cylinder of woody tissue. No pits could be distinguished on the tracheids. According to GOTHAN (4), the tracheids of wood associated with *Araucarites windhausenii* bear but a single row of closely spaced bordered pits, a situation not characteristically Araucarian.

The cone scales measure 20 mm. in length and are about 14 mm. broad at the distal end. They retain this width almost to the point of divergence from the cone axis, where they narrow to about 10 mm. Each bears in a median position a single totally imbedded seed, which occupies slightly more than the proximal half of the scale length. The cone scales differ from those described by GOTHAN (4) and SPEGAZZINI (12), in that these have prominent wings throughout their entire length (fig. 10, B).

The relative proportions of the cone scale are shown in figures 5 and 6. The structure parallels so closely that of the scales of modern Araucarians, especially those of the Eutacta section, that *Proaraucaria mirabilis* furnishes no added evidence to settle the controversy centered about the status of the carpellate cone scales in *Araucaria*. The structure of the vascular bundles of the scale also is not sufficiently well preserved to show their orientation, although the eroded apices of some of the scales show the presence of several vascular strands. Cell structure is unusually well preserved at the fleshy dis-





FIGS. 5-7.—Fig. 5, diagram of median longitudinal section of cone scale (*b*, bract; *l*, ligule; *i*, integument; *g*, megagametophyte; *e*, embryo; *n*, nucellus; *m*, micropyle). Fig. 6, surface view from above of cone scale. Fig. 7, *A*, median longitudinal section of embryo showing cotyledons and hypocotyl; stem tip indicated at *a*. *B*, *C*, *D*, *E*, transverse sections of embryo showing vascular structure taken at planes *b-b*, *c-c*, *d-d*, and *e-e* on longitudinal diagram, fig. 7 *A*.

tal end of the scale. Adaxially from the chalazal end of the seed, however, the preservation of the cell structure of the interior tissues of the scale fails, only an exterior border four or five cells deep being present. The heavy walls indicate that the scale may have been woody.

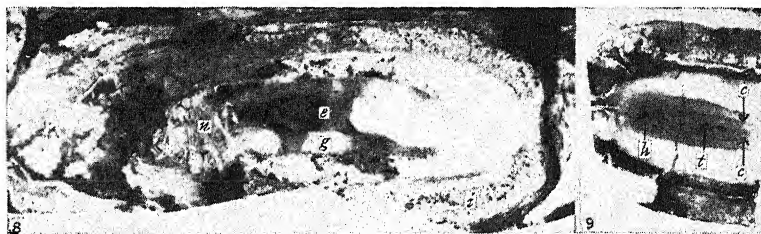
Because most of the recorded fossil Araucarian cone material consists of isolated cone scales lacking associated leaf material, it has little value for comparison with the present fossil. If the cone scales of *Proaraucaria mirabilis* had been found isolated, they would undoubtedly be classified in the Eutacta section of the genus on account of their broad wings and prominent ligules. The association with the cones of twigs bearing the broad leaf scars of the Colymbea type, however, as well as the fact that the embryo has two cotyledons, raises the question of their exact affinity. *Araucarites microphylla* Saporta, from the Jurassic of France (8), has in association with its Eutacta type scales, leaves of the Colymbea type, similar to those of *Araucaria bidwillii* Hook. but smaller. The North American *Araucarites hespera* Wiel. (14), which has Eutacta scales, has been found associated with *Araucarites hatcheri* Wiel. (14) which has Colymbea type leaves, and may represent a second example of Eutacta scales and Colymbea leaves in the same plant.

Among modern forms the cone scales of *Proaraucaria mirabilis* may be most closely compared with those of *Araucaria bidwillii*, which have broadly winged scales bearing prominent ligules, Colymbea leaves, and an embryo having two cotyledons. The latter is regarded as a transitional form between the Eutacta and the Colymbea sections, but is classified as a Colymbea form because of its leaves, its two cotyledons, and the hypogeal germination of its seeds. The points of difference from *Proaraucaria mirabilis* are its much larger cone size and the presence of a long spine at the scale tip.

The two living South American species, *Araucaria angustifolia* (Bertol.) Ktze. and *A. araucana* Koch., are characterized by having much reduced scale wings, the former species having none at all. In addition, both have relatively inconspicuous ligules, Colymbea leaves, two cotyledons, and hypogeal germination. This reduction in the modern South American species may well represent development from types such as *Proaraucaria mirabilis*, which were present

and dominant in the same geographic locality in past ages, specialization accompanied by isolation finally resulting in the modern type. Curiously enough, it is only the South American species which exhibit this reduction in scale wings. Also, this seems to be a comparatively recent development, as none of the fossil scales found in any part of the world are of this reduced type.

SEED.—The seeds of cone 13854 average 11 mm. in length. They are somewhat flattened, so that the diameter parallel to the scale measures about 6 mm. at the broadest region, while the diameter at right angles to the scale seldom exceeds 4 mm. The integument



FIGS. 8, 9.—Fig. 8, longitudinal section of seed, cut so as to remove one cotyledon of embryo (*i*, integument showing thin area at chalazal region; *n*, nucellus showing wavy nature as if disintegrating or crushed; *g*, megagametophyte; *e*, embryo). Fig. 9, median longitudinal section of embryo showing both cotyledons (*c*), stem tip (*t*), and hypocotyl (*h*). Tips of cotyledons have been removed. Vascular strands may be traced from cotyledons to hypocotyl.

seems to be composed of three layers, an outer and inner thin layer and a much heavier, fibrous, middle layer. The thick middle layer thins abruptly at the chalazal region. Closely appressed to the inner surface of the seed coat is a thin layer of nucellar tissue. It must have shrunk markedly before petrification, leaving a lumen which was subsequently filled with transparent quartz. At the micropylar end of the seed the nucellar tissue is more abundant, and extends into the micropylar tube. Here it has a wavy appearance, as if the living tissue had been partially digested or crushed.

The tissue of the megagametophyte has been replaced by a dense ivory colored silica, devoid of cell structure. It is massive, measuring 2.5 to 3 mm. by 1 to 1.5 mm. in cross section, and having a length of 5.5 to 6 mm.

Notwithstanding their small size, about 5 mm. in length and only

1 mm. in diameter, the embryos are mature or approximately so. They extend almost the full length of the megagametophyte, and have two well defined cotyledons and a well developed hypocotyl.

In the median longitudinal section, represented diagrammatically in figure 7 *A*, the cotyledons are seen to diverge slightly below the middle of the embryo. The relatively flat stem tip is indicated at *a*.

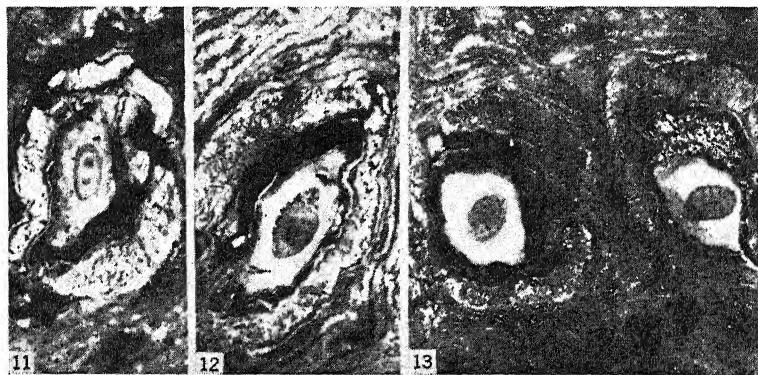


FIG. 10.—Transverse sections of several seeds cut at various planes. Section at *A* cuts embryo near base of hypocotyl, showing diarch stele. Seed at *B* cut through nucellar region below base of hypocotyl near to cone axis. Tips of the broad scale wings at this adaxial location are indicated by the two arrows.

*B*, *C*, *D*, and *E* of figure 7 represent diagrams of transverse sections taken at *b-b*, *c-c*, *d-d*, and *e-e* respectively on the longitudinal diagram. The vascular pattern is as follows: At the base of the hypocotyl (*E*) the stele is diarch, and is represented by a lens-shaped centrally located mass. Farther up, at *D*, which represents a section slightly above the cotyledonary plate, the vascular strands are grouped in two crescent-shaped masses. At *C* the section passes through the embryo just above the stem tip, as is indicated by the presence of the circular opening at the center. Here the two cotyledons are distinct and the vascular bundles of the cotyledons are

distinguishable. The plane of the cut in *C* was not exactly at right angles to the perpendicular axis of the embryo, so that the cotyledon on the left has been cut at a slightly higher level than that on the right, and consequently shows a greater number of bundles. The cross section at *B* is at a more distal plane in the cotyledons, where the full number of six bundles in each cotyledon is distinct.

Since the diamond saw removes with each cut more than 1.5 mm. of material, it is obvious that not enough serial sections could be



FIGS. 11-13.—Fig. 11, transverse section of seed showing embryo cut through region of cotyledonary plate. Vascular tissue represented by two crescent-shaped masses. Numerous resin ducts visible below epidermal region. Fig. 12, embryo cut just above point of divergence of cotyledons. Space just above stem tip shown at center. Cotyledon above shows presence of four bundles, lateral pair on point of diverging to form a total of six. Cotyledon below shows two masses of vascular tissue, each about to divide. Resin ducts may also be seen below epidermal region. Fig. 13, transverse section of two seeds cut at more distal plane of embryo; two cotyledons distinct in both embryos, each having six vascular strands. Resin ducts also visible.

obtained from any single embryo to supply all the data necessary for the reconstruction. Figure 7 therefore represents a hypothetical reconstruction from observations made on several embryos.

The embryo of *Proaraucaria mirabilis* is in general very similar to those of modern species of the Colymbea section of the genus *Araucaria*, both in exterior aspect and in vascular anatomy. In the modern species, *Araucaria araucana* and *A. angustifolia*, the hypocotyl constitutes less than one-sixth of the total length of the embryo, while in the fossil it comprises about one-half.

The vascular pattern corresponds closely with that of *Araucaria*

*araucana*. A minor difference is that the number of cotyledonary bundles in the modern form is variable, ranging from six to eight, while never more than six were observed in the fossil. The vascular structure also resembles closely that of *A. angustifolia* discussed by HILL and DE FRAINE (6), and *A. bidwillii* given by SHAW (11).

### Summary

1. The cones of *Proaraucaria mirabilis* show the most excellent preservation of any fossil *Araucaria* cones thus far recorded.

2. Associated with them are other portions of the plant, including twigs bearing the broad leaf scars typical of *Colymbea* leaves.

3. The cone scales have the characteristics of those of the *Eutacta* section of the genus *Araucaria*, that is, broad scale wings and prominent ligules, but the fossil is considered a *Colymbea* form because of its broad leaves and because its embryos have two cotyledons.

4. *Proaraucaria mirabilis* thus resembles the fossils *Araucarites microphylla* Sap. and *A. hespera* Wiel., which had *Eutacta* cone scales and *Colymbea* leaves.

5. *Proaraucaria mirabilis* is to be most closely compared with *Araucaria bidwillii*, the only living species which combines winged scales with broad *Colymbea* leaves. The cones of *A. bidwillii*, however, are much larger than those of the fossil.

6. The embryos of *Proaraucaria mirabilis* are in general similar, although much smaller than those of living species of the *Colymbea* section. Their chief difference, aside from their small size, is that they have a proportionately much larger hypocotyl than the living forms.

7. The gross vascular anatomy corresponds closely to that in *Araucaria araucana*, *A. angustifolia*, and *A. bidwillii*.

The writer expresses appreciation to Dr. A. C. NOÉ, under whose direction the study was conducted, and to the Field Museum of Natural History for lending the specimens for study. Dr. G. W. GRAVES of State Teachers College, Fresno, California, Dr. L. L. BURLINGAME of Leland Stanford University, and Dr. P. F. SOUSA of Rio de Janeiro kindly furnished seed and cone material of living *Araucarians*.

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# MORPHOLOGICAL AND CULTURAL STUDIES OF *TAPHRINA POTENTILLAE*<sup>1</sup>

ELLA MAY MARTIN

(WITH ELEVEN FIGURES)

## Introduction

Early descriptions of *Taphrina potentillae* Farw. Johanson give briefly the morphology of the fungus and the symptoms shown by the host, *Potentilla canadensis*, but apparently there has been no report of cultural or inoculation experiments with this fungus. FARLOW (2), JOHANSON (6), and ROBINSON (14) measured and described the ascus. JUEL (7) described and figured the ascogenous cell and the young ascus.

## Morphological studies

MATERIALS AND METHODS.—Leaves of *Potentilla canadensis* infected with *Taphrina potentillae* were collected and fixed early in July, 1928, near Ithaca, New York, and the following May at Greensboro, North Carolina. At Greensboro, plants had been successfully inoculated with cultures isolated at Ithaca. Gilson's and Flemming's weaker fixing solutions proved to be the best for a study of the fungus. Material was dropped into Carnoy's solution for one minute before it was placed in the other fixing solutions. This method gave more satisfactory results than did the use of an air pump. By fixing material in the field immediately after collection there was the least plasmolysis. Paraffin sections were cut from 2 to 5  $\mu$  in thickness. They were stained with Flemming's triple stain and by the anilin oil gentian violet method as used by Claussen.

SYMPTOMS AND EFFECTS ON HOST.—Circular or elongated blisters are produced on the leaflets of *Potentilla canadensis* infected with *Taphrina potentillae*. The infected areas become convex on the upper surface and concave on the lower, and vary in length from 1 mm. in

<sup>1</sup> A part of this paper was presented before the American Phytopathological Society at Cleveland in 1930; an abstract was published under the title: Cultural and inoculation experiments with *Taphrina potentillae*. *Phytopath.* 21:121. 1931.



their early stages to 1 cm. when older. When first noticeable they are yellow, but later they become purple and brown. Lesions are most frequent near the lateral margins and tips of the leaflets (fig. 1), but some are located near their bases. Swelling of the petiole described by JOHANSON, when found in this material, is very slight.

The leaflets are not distorted by this fungus so much as are the leaves of the peach when infected with *Taphrina deformans*. In *Po-*

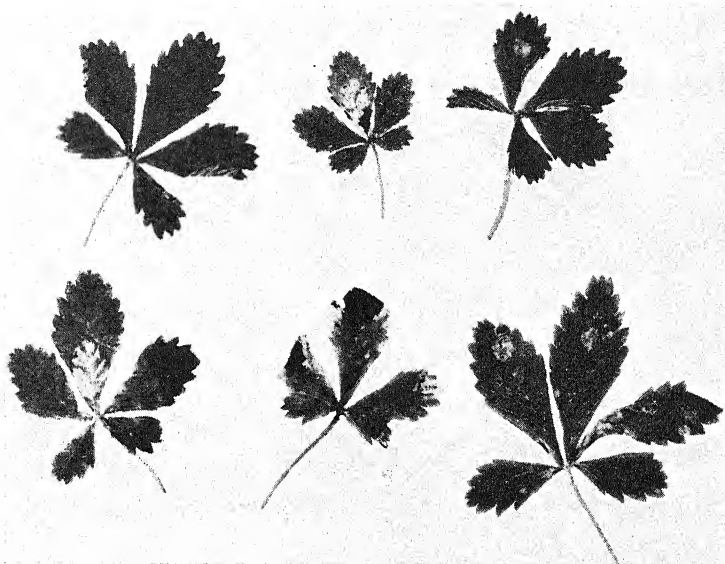


FIG. 1.—Typical lesions produced by *Taphrina potentillae* on leaves of *Potentilla canadensis*; each leaf inoculated with a different combination of single spore cultures.

*tentilla canadensis*, the diseased areas, which are only small blisters, are thicker than normal parts. This thickening and blistering is due to an increase in the number of cells in the palisade and upper portions of the spongy parenchyma.

MORPHOLOGY OF FUNGUS.—The form of the vegetative and the ascogenous cells of *Taphrina potentillae* is largely determined by the host cells surrounding them. The vegetative cells may vary from ovoid to cylindrical as they grow between the mesophyll cells of the leaf. Both the vegetative and the young ascogenous cells are binucleate (figs. 2-4). The latter are frequently ovoid and are scattered

irregularly beneath the epidermal layer. Their position beneath the epidermis was early mentioned by GIESENHAGEN (4) and SADEBECK (15) for this species of *Taphrina* and for *T. flava*. In all other species of *Taphrina* the ascogenous cells form a definite layer between the cuticle and the epidermis.

As the ascogenous cells grow between the epidermal cells to form asci, they become very slender, but after they have broken the cuticle and have grown beyond the surface of the leaf, they broaden considerably. Thus the asci which are found on both surfaces of the leaf are clavate, with their pedicels remaining between the epidermal cells.

As the ascogenous cell develops into an ascus, the two nuclei in it fuse (fig. 5), and the fusion nucleus, according to JUEL, soon undergoes division. Two successive mitotic divisions have been described by JUEL for this species. It is assumed that three successive divisions occur in the developing ascus, thus forming the eight nuclei for the primary ascospores.

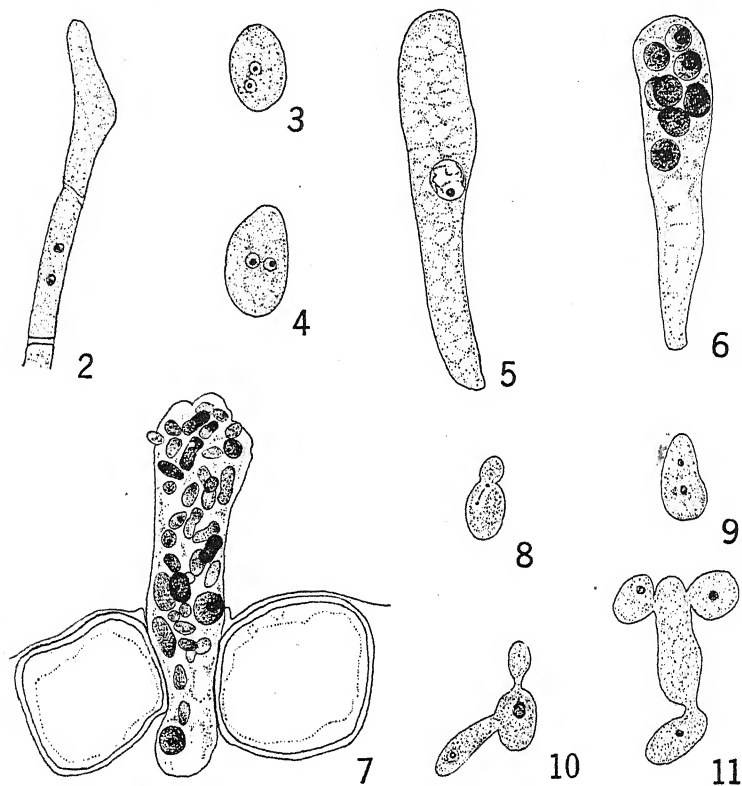
Eight primary ascospores are commonly found in the ascus (fig. 6), but before the spores are discharged budding takes place, resulting in a large number of minute spores in the ascus (fig. 7). Occasionally one of the large ascospores may continue without budding up to the time of spore discharge. Ejection of the spores occurs at the apex of the ascus, leaving an irregularly broken margin.

### Cultural studies

METHODS OF ISOLATION.—Diseased portions of leaves were fastened to the covers of petri dishes so that the asci could discharge their spores upon the agar below. After one-half hour, the petri dishes were inverted and individual spores were marked by using an 8 mm. objective and a 12 or 15 $\times$  ocular. The media used were clear Harter agar made without peptone, potato dextrose agar, and a dextrose agar to which juice from *Potentilla* leaves was added. The pH of these agars ranged from 4.5 to 5.

Spores of this species of *Taphrina* are extremely small when they are discharged, their dimensions often averaging  $2.5 \times 3\mu$ . Their transparency also makes the marking of them a tedious process. This difficulty was overcome by using a clear potato dextrose agar

to each liter of which 0.1 gm. of Congo Red had been added. Congo Red is readily absorbed by *Taphrina* spores but does not hinder their growth. The petri dishes were next placed in a constant temperature



FIGS. 2-11.—Fig. 2, vegetative mycelium of *Taphrina potentillae* within leaf of *Potentilla canadensis*; figs. 3, 4, binucleate ascogenous cells; fig. 5, young ascus containing fusion nucleus; fig. 6, eight spored ascus; fig. 7, many spored ascus; figs. 8-11, stages in budding of spores on Sabouraud's agar.

chamber at 9° C. for twelve hours or a little longer. It had been found previously with *Taphrina coryli* (9) that chilling the spores for twelve hours and then bringing them up to room temperature would hasten germination. Spores germinated rapidly after one-half hour at room temperature, and having absorbed Congo Red, were easily marked. Only the more isolated of these germinating spores were

marked, and colonies developing from them were transferred to potato dextrose agar slants. Thus twenty-eight single spore cultures of *T. potentillae* were obtained. In cases where spores were not well scattered, dilution plates were poured. The color absorbed from the Congo Red was retained by the organism for some time, but after several transfers to standard potato dextrose agar without Congo Red, the cultures became pale pink in color and similar to those which were isolated on ordinary potato dextrose agar. The latter cultures are a grayish pink, opaque, and glistening, and are very much like agar cultures of *T. johansonii* described by the writer (10) and *T. deformans* sent to the writer by MIX and described by him (12). Compared with the colors given in Ridgway's Color Standards and Color Nomenclature, cultures of *T. potentillae* might be described as "pale vinaceous fawn."

INOCULATION EXPERIMENTS.—The agar cultures which were isolated at Ithaca were taken to Greensboro, where they were used the following spring for inoculating plants of *Potentilla canadensis*. No infected plants had been found in that region and none had been reported nearer than Durham, North Carolina, 50 miles away. The plants selected for experimental work were growing 4 to 6 feet apart. Since the hillside on which they grew was burned over each February, all plants available for inoculation had leaves of only the current season's growth.

Inoculations were always carried on in the late afternoon during a light rain. Young leaves were selected on each plant and a small amount of potato dextrose agar with the spores was spread on the upper and lower surfaces of each leaflet. Before and after inoculating, the plants were sprayed with sterile distilled water. Plants were covered with fine meshed cheesecloth or muslin well elevated over a framework.

Following the method just described, thirty-eight plants were inoculated March 26 and 27, 1929. Eleven single spore cultures were selected for use. Assuming that these cultures might be plus or minus, five of them were used alone and these and the others in different combinations. By May 11 of that year, 50 per cent of the plants showed characteristic blisters. The amount of infection was the same whether plants were inoculated with one culture or with a

combination of cultures. Seventeen of the thirty-three plants inoculated with combinations of cultures and two of five plants inoculated with single spore cultures became infected. The lesions on leaves shown in figure 1 were produced by combining different cultures.

The next spring, eight of the same cultures were used in inoculating sixteen other plants which were located a quarter of a mile away from the plants used previously. This time 75 per cent of the plants showed infection even though eight of them were inoculated with single spore cultures and the other eight with combinations of cultures. Six of each group of plants showed the blisters characteristic of plants infected by *Taphrina potentillae*.

Plants infected as a result of inoculations during these two years did not show infections in later years. Since the areas where they were growing were burned over each February, spores were evidently killed, for the new leaves coming from the rhizome were not infected.

Climatic conditions evidently have much influence upon the amount of infection resulting from inoculation. Plants inoculated in the autumn with the same cultures showed no infection although some were grown all winter out of doors under muslin and others were kept in the laboratory covered with bell jars. Negative results were also obtained with leaves inoculated at the same time and kept in moist sterile petri dishes.

**SPORE GERMINATION AND CONJUGATION.**—Spores of *Taphrina potentillae*, when discharged from the ascus, continue to carry on their yeast-like budding, either on the surface of leaves of the host or on the media mentioned.

All workers are in agreement on the budding of ascospores of *Taphrina* species, but there are decided differences of opinion as to whether conjugation takes place. KLEBAHN (8) and WIEBEN (16) described conjugation for some species of *Taphrina*. In these species they hold that the two nuclei present in the cells of the vegetative mycelium result from the conjugation of spores or of young hyphae. JANKOWSKA (5), however, working with *T. sadebeckii*, found no conjugation. The writer (11) reported that spores of *T. johansonii* and *T. deformans* did not conjugate on artificial media. FITZPATRICK (3)

saw no cases of conjugation in *T. deformans*, but MIX (12) found occasional, although incomplete, conjugation in the same species.

With these results in mind, the first work done by the writer was to grow single spore cultures and combinations of these cultures, as reported in 1931 (footnote 1). The cultures were grown in Van Tieghem cells in drops of the following media: cleared potato dextrose agar, Sabouraud's agar, a decoction made from *Potentilla* leaves, and a dextrose agar made with the juice from *Potentilla* leaves. The hydrogen-ion concentration of these types of media was from 5.4 to 5.9. This material was fixed in Juel's or in Bouin's fixing solution, stained with iron-alum haematoxylin or Flemming's triple stain, and mounted in Venetian turpentine or balsam. Similar material was removed from inoculated leaves and treated in the same manner.

The slides made previous to the report in 1931 were re-examined during the present year. It is now seen that cases which appeared to be conjugation (figs. 10, 11) are rare, and in no case have the spores which are in contact shown a complete movement of the protoplast from one spore to the other. Budding, however, is abundant and is always preceded by nuclear division (figs. 8, 9). This nuclear division in the budding spore produces the binucleate condition of the young mycelium.

### Discussion

Spores and young hyphae which appear to be conjugating are found so seldom that they cannot account for the presence of two nuclei in the cells of the young mycelium. Such apparent conjugation may represent only irregularities in budding, or chance contact of spores; or in young germ tubes it may be merely the anastomosing of hyphae. The latter relationship, if it does exist, evidently does not constitute a definite part of the life cycle of the organism.

From these investigations, showing that either single spore cultures or combinations of them may produce infection, it is assumed that *Taphrina potentillae* is a homothallic form. In this species the dicaryophase is evidently initiated by division of the nucleus in the spore at the time of germination, as suggested by EFTIMIU (1) and FITZPATRICK (3) for *Taphrina deformans*, and by the writer (11) for this species and *T. johansonii*.

### Summary

1. *Taphrina potentillae* produces circular or elongated blisters on the leaflets of *Potentilla canadensis*. The ascogenous cells of this fungus are produced between the epidermis and the mesophyll, but they grow between and beyond the epidermal cells to form asci with slender pedicels. Eight primary spores are formed in the ascus but these bud before they are discharged, forming numerous minute spores.

2. Spores isolated on potato dextrose agar form colonies which are grayish pink, opaque, and glistening.

3. Plants inoculated either with combinations of the eleven single spore cultures or with only single spore cultures showed infection.

4. Stages which appear like conjugation of spores are rare and do not represent the union of protoplasts, so this is not now considered conjugation.

5. Spores of *Taphrina potentillae* bud to form short chains and then hyphae, either on artificial media or on the surface of the host. Nuclear division in the budding ascospore initiates the dicaryophase.

This study was completed while the writer had guest privileges in the Department of Botany at the University of Chicago.

UNIVERSITY OF CHICAGO

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# CYTOLOGICAL INVESTIGATIONS OF ERECHTITES HIERACIFOLIA

GEORGE OLDS COOPER

(WITH THIRTY-SEVEN FIGURES)

## Introduction

Aside from the single genus *Senecio*, few cytological investigations have been made in the Senecioneae. GUIGNARD (7) briefly described the mature embryo sacs of *Petasites* and *Tussilago*. AFZELIUS (1), CARANO (4), DAHLGREN (5), and SMALL (8) found a normal type of development for the megagametophyte in *Senecio* but laid major emphasis on the behavior of the antipodal cells. AFZELIUS showed a multinucleate condition in the antipodals of *Cacalia suaveolens* and *C. reformis*. SMALL found a similar condition in *Tussilago* and *Petasites*. He likewise described microsporogenesis and the development of the anther in *Senecio vulgaris*. SOUÈGES made a detailed study of the development of the embryo in this species.

## Material and methods

The material of *Erechtites hieracifolia* (L.) Raf. was gathered on the campus of the University of Wisconsin. The investigation was carried on through the facilities of the Departments of Botany and Genetics. Since the flowers are produced in a slowly maturing head, buds were collected at various stages of development. They were fixed either in Carnoy's solution or in Karpechenko's modification of Navashin's solution, imbedded in paraffin, and cut 12-14  $\mu$  in thickness. They were stained either in Delafield's haematoxylin or in Smith's modification of Gram's iodine-crystal-violet. The latter was found to give better differentiation in material at early stages of development. All drawings were made with an Abbé camera lucida at table level.

## Investigation

MICROSPOROGENESIS.—Each theca of the anther contains a single row of microspore mother cells which are considerably larger than the surrounding vegetative cells. From five to seven such cells are found in each row (fig. 1). As the microspore mother cells approach

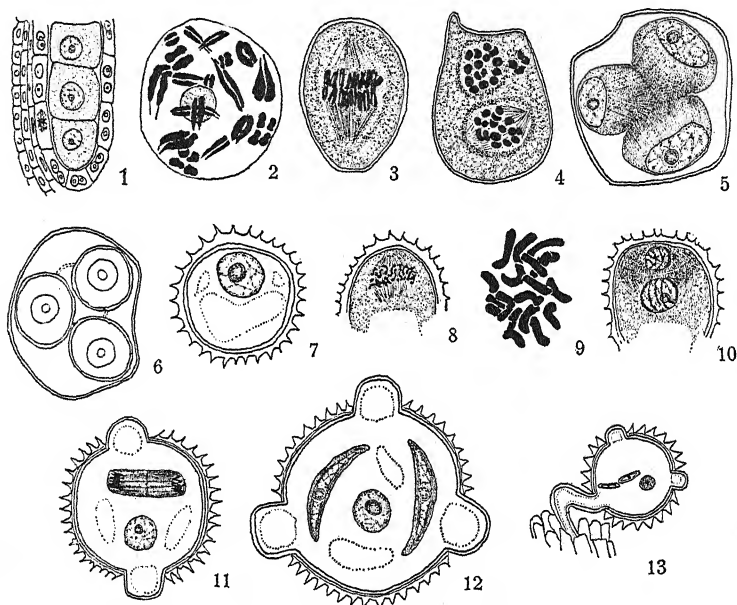
synizesis, mitotic divisions occur in the tapetal cells so that each cell is binucleate at maturity. At diakinesis 20 pairs of chromosomes are present (fig. 2). AFZELIUS has reported the same number for *Senecio vulgaris*, whereas SMALL found 19 to be the gametic number. A marked enlargement of the microspore mother cell occurs during the period in which the nucleus is passing through the late prophase stages. At metaphase of the heterotypic division (fig. 3) each of the chromosomes is split longitudinally. The spindles of the homoeotypic division are usually at right angles to each other (fig. 4), but occasional cells were found wherein they were parallel. Twenty chromosomes were present at the homoeotypic division (fig. 4). A somewhat denser zone of cytoplasm surrounds the spindles of both the heterotypic and homoeotypic divisions (figs. 3, 4). These were particularly pronounced at the homoeotypic equatorial plate stage. The second peripheral zone of cytoplasm observed by D. C. COOPER (2) in *Portulaca oleracea* and by the writer (3) in the Chenopodiaceae was not observed.

As a result of the homoeotypic division, a cell containing four nuclei is formed. The spindles of the meiotic divisions persist for a time and cell plates are laid down across their mid-regions (fig. 5). In the process of spore formation splits occur along these cell plates, an observation which is at variance with that of GATES and REES (6) who state the spores are formed as a result of furrowing.

The newly formed microspores are held together for a time in a matrix of stainable material. The microspore increases in size and an irregular spiny exine is developed (fig. 7). The intine appears shortly after the division of the microspore to form a two celled gametophyte.

The microspore nucleus divides to form the generative nucleus and the tube nucleus (fig. 8). A cell plate is laid down across the spindle and the microspore is divided to form a large tube cell and a smaller generative one (fig. 10). The method of procedure is similar to that noted by the writer as occurring in the Chenopodiaceae. The elongated generative cell ultimately becomes deeply imbedded in the cytoplasm of the tube cell and its limiting membrane is scarcely perceptible except in thin sections. Shortly before anthesis the generative cell divides to form two male gametes. The nuclear division is typically mitotic and at telophase a cell plate is formed across the

mid-region of the spindle (fig. 11). The two male gametes exist as distinct cells which become much elongated and sickle-shaped as they approach maturity (fig. 12). Each pollen grain has three germ pores more or less equally spaced on its surface (fig. 12). Each of these pores contains a large vacuole and is covered with a thin intine.



FIGS. 1-13.—Microsporogenesis: fig. 1, longitudinal section through anther showing single row of spore mother cells; fig. 2, diakinesis, 20 pairs of chromosomes; fig. 3, heterotypic metaphase; fig. 4, homoeotypic metaphase, spindles at right angles; fig. 5, cytokinesis, thickenings on spindles and partition walls beginning to advance across spindle; fig. 6, four spores in matrix of old microspore mother cell; fig. 7, mature microspore; figs. 8-10, stages in division of microspore nucleus; fig. 11, telophase in division of generative cell; fig. 12, mature pollen grain; fig. 13, germinating pollen grain.

The exine is lacking in this region. Shortly after the pollen comes in contact with the stigmatic surface, the pollen tube emerges at one of the germ pores and passes between the cells of the stigma (fig. 13). The male gametes and the tube nucleus remain in the pollen grain for some time after the formation of the pollen tube. Ultimately they likewise pass into the pollen tube and along it to the micropylar end of the megagametophyte.

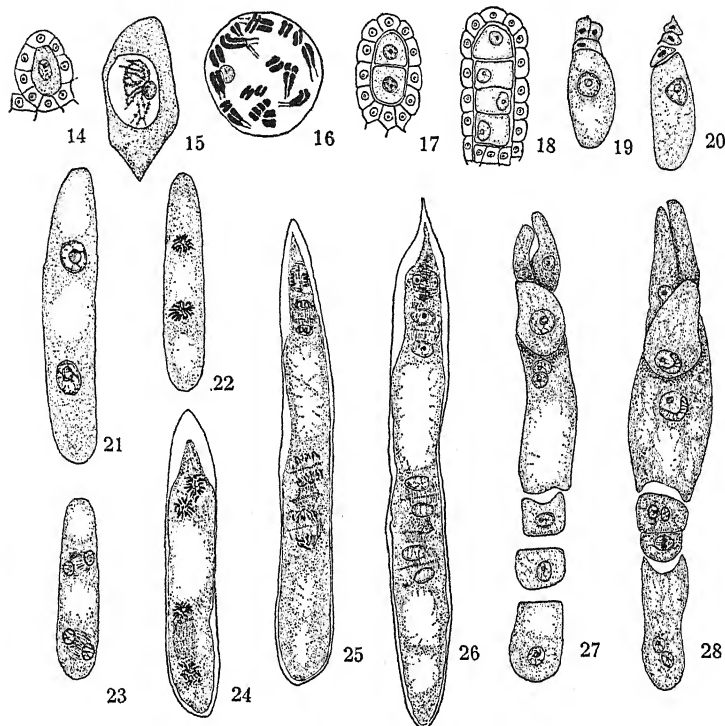
**MEGASPOROGENESIS.**—The single archesporial cell is hypodermal

and functions directly as the megaspore mother cell (fig. 14). During the process of the development of the ovule the megaspore mother cell grows both in length and width. Its nucleus likewise enlarges while passing through the stages leading to the diakinesis stage of the heterotypic division. The cytoplasm of the cell remains finely vacuolate. Twenty pairs of chromosomes are present at diakinesis (fig. 16). Following the heterotypic division two cells are formed (fig. 17), and subsequent to the homoeotypic division a linear row of four megaspores (fig. 18) results. Shortly after the formation of the megaspores, the chalazal one elongates and the other three disintegrate (figs. 19, 20). SMALL likewise found that the basal cell functions in *Senecio vulgaris* and that the three micropylar spores disintegrate.

The functional megaspore nucleus divides to give a two nucleate megagametophyte (fig. 21). A large vacuole develops in the mid-region of the cell between the two nuclei. As a result of the two further divisions an eight nucleate megagametophyte is formed. In the course of the last division of the nuclei, the spindles of the two chalazal nuclei are end to end so that a linear row of four is formed. The two spindles at the micropylar end are at right angles to each other, the longitudinal axis of the apical spindle being transverse to the longitudinal axis of the megagametophyte. Cell plates are formed across the spindles of the last division as well as across the persistent spindles of the preceding division in such a manner as to form three uninucleate cells at each end of the megagametophyte, and the polar nuclei one from each end remain in the large central cell (figs. 25, 26). The completed eight nucleate, seven celled megagametophyte consists of a linear row of three antipodals, a primary endosperm cell containing two nuclei, a pear-shaped egg cell and two club-shaped synergids. The basal antipodal cell is about twice as large as either of the other two (fig. 27). The cytoplasm of the synergids is finely vacuolate in the basal region near the egg cell and elongate striations occur in the apical region. The cytoplasm of the megagamete is finely vacuolate until just prior to fertilization.

Double fertilization occurs, one male gamete uniting with the primary endosperm nucleus formed as a result of the union of the two polar nuclei, the other uniting with the egg nucleus (fig. 28).

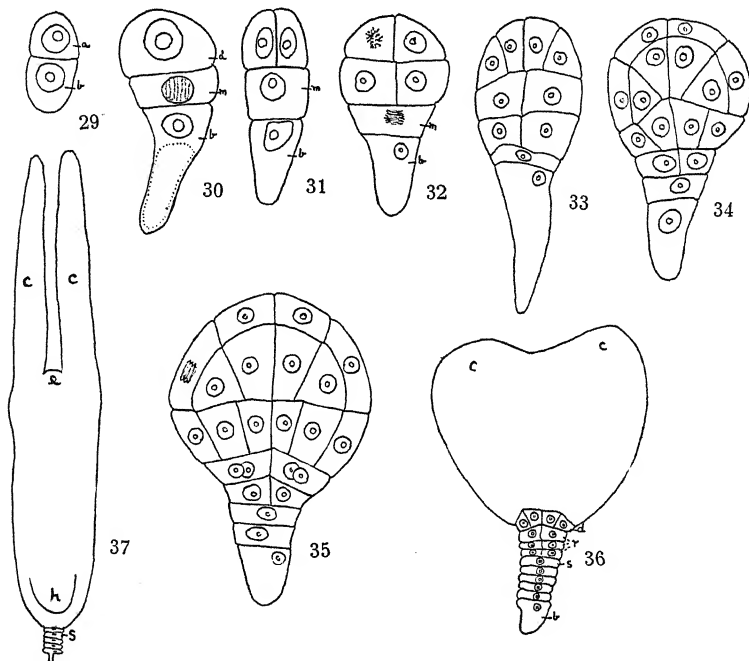
The cytoplasm of the egg cell develops elongate vacuoles in the apical region. The antipodal cells are usually binucleate at the time of fertilization.



FIGS. 14-28.—Megasporogenesis: fig. 14, megaspore mother cell; fig. 15, same, nucleus at synizesis; fig. 16, diakinesis, 20 pairs of chromosomes; fig. 17, interkinesis; fig. 18, linear row of four megaspores surrounded by nucellus; figs. 19, 20, functional megaspore with three disintegrating spores; figs. 21-28, stages in development of megagametophyte: fig. 21, two nucleate stage; fig. 22, metaphase preceding four nucleate stage; fig. 23, early four nucleate stage; fig. 24, metaphase preceding eight nucleate stage; figs. 25, 26, cell plate formation; fig. 27, mature megagametophyte, uninucleate antipodals; fig. 28, double fertilization, antipodals binucleate.

In some instances the antipodals remained uninucleate even after fertilization, and in no instance was a multinucleate antipodal observed. AFZELIUS found the antipodals of the forms he studied to be either binucleate or multinucleate. He states that in some instances the antipodals may divide so that instead of having typically three

antipodal cells, a larger number may be present. SMALL records that the chalazal antipodal may divide to form as many as four cells, each cell having one or more nuclei. This elongated structure is spoken of as an aggressive haustorium. Such variations in antipodal formation were not observed in *Erechtites*.



FIGS. 29-37.—Development of embryo: Fig. 29, two celled proembryo; *a*, apical cell; *b*, basal cell. Fig. 30, three celled proembryo; *a'*, apical cell; *m*, middle cell; *b*, basal cell. Fig. 31, two celled embryo with two celled suspensor. Fig. 32, four celled embryo, middle cell of suspensor dividing. Figs. 33-35, further development of embryo. Fig. 36, first evidences of cotyledons, *c*; dermatogen, *d*; root cap, *r*. Fig. 37, mature embryo; *e*, epicotyl; *h*, hypocotyl; *s*, suspensor.

DEVELOPMENT OF EMBRYO.—The zygote divides to form a two celled proembryo with cells about equal in size (fig. 29). The apical cell divides so that a row of three cells is formed (fig. 30), consisting of an elongated basal cell (*b*), a somewhat flattened middle cell (*m*), and a rounded terminal cell (*a'*). The terminal cell becomes greatly enlarged and then divides vertically to form the two celled embryo at the apex of a two celled suspensor (fig. 31). Later a transverse di-

vision of these two cells produces a four celled embryo (fig. 32). In this development *Erechtites* differs from *Senecio* wherein the middle cell of the three celled stage divides first and the distinct quadrant of cells is lacking (9). Further development of the embryo is comparable in the two genera. Periclinal divisions of the quadrant cut off the dermatogen. Later the periblem and plerome are delimited and in the process of further differentiation the embryo becomes spherical (figs. 34, 35). As a result of active cell division the embryo elongates and becomes flattened at the apex. Shortly thereafter the primordia of the cotyledons appear. This is in agreement with that shown in other Compositae. The basal and suspensor cells now show signs of disintegration. The cotyledons elongate further, the epicotyl appears, and the cyclic arrangement of the cells at the base of the elongating hypocotyl shows the beginning of the root cap (fig. 37).

### Summary

1. Each theca of the anther of *Erechtites hieracifolia* contains a single row of five to seven microspore mother cells.
2. The heterotypic and homoeotypic spindles are surrounded by a dense sheath of granular cytoplasm.
3. Paired thickenings appear on the cytoplasmic strands midway between the nuclei of the tetrad, and the four spores are formed as the result of these cell plates.
4. The spore divides to form a two celled pollen grain.
5. The generative cell becomes deeply imbedded in the cytoplasm of the tube cell and then divides to form two male gametes.
6. A single megaspore mother cell is found in the ovule of each flower.
7. As a result of meiosis a row of four megaspores is formed.
8. The chalazal megaspore develops into an eight nucleate, seven celled megagametophyte, and the other megaspores disintegrate.
9. Double fertilization occurs.
10. At the time of fertilization the antipodals are usually binucleate.
11. A proembryo of three cells is formed.
12. The embryo arises as a result of further divisions of the apical cell of the proembryo.

13. The suspensor is formed from the two basal cells of the pro-embryo.

14. The haploid chromosome number is 20.

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## CYTOLOGY OF CERTAIN LABIATAE

ELIZABETH P. BUSHNELL

(WITH TWENTY-TWO FIGURES)

Only a few genera of Labiatae have been investigated from the standpoint of chromosome number and chromosome morphology. The haploid numbers in *Galeopsis* are reported by MÜNTZING (3) to be eight and sixteen. JÖRGENSEN (2) made chromosome counts of ten species of *Lamium*, seven of which had the haploid chromosome number of nine; in the others the number was eighteen. SCHEEL (6) determined the chromosome numbers in species of *Rosmarinus*, *Scutellaria*, *Lavandula*, *Sideritis*, *Coleus*, and *Salvia*. RUTTLE (4) found two species of *Mentha*, *M. requienii* with nine chromosome pairs and *M. pulegium* with ten, that differ in morphological character as well as in chromosome number from other members of the genus. Other species of *Mentha* form a twelve-chromosome series. The diploid number in three species of *Lycopus* RUTTLE (5) determined to be twenty-two. HRUBY (1) reported sixteen as the haploid number in three species of *Prunella*.

For the present study, flower buds of *Monarda fistulosa*, *M. didyma*, *M. punctata*, and *Nepeta cataria* were fixed with Navashin's, Licent's, and Flemming's fluids. The material was sectioned at 10-15  $\mu$ ; sections 13  $\mu$  in thickness included whole nuclei and were most valuable for study. Stains used were Newton's iodine-crystal violet, Flemming's triple, and Heidenhain's iron-alum haematoxylin.

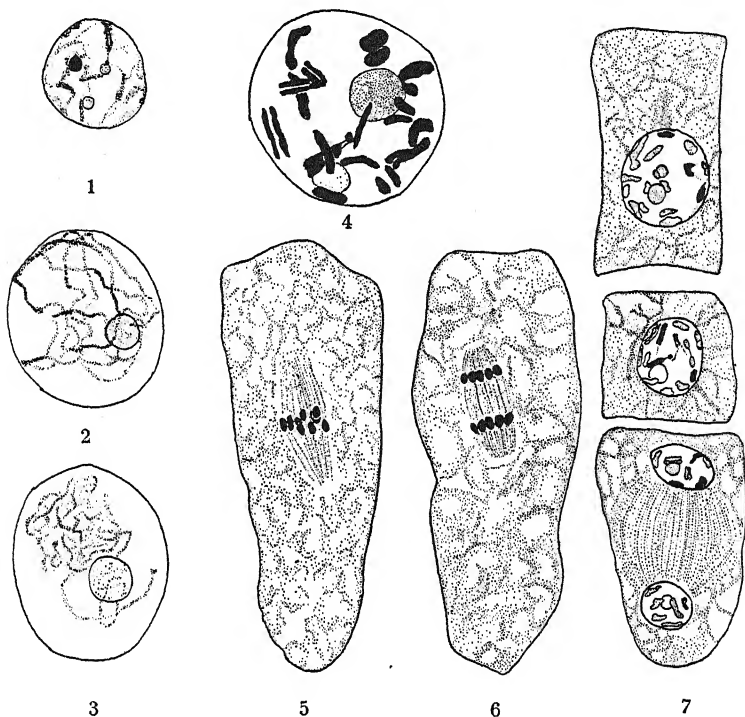
### Observations

#### *Monarda fistulosa*

At first the macrospore mother nucleus is small, containing one to three nucleoli and chromosome-like bodies connected by fine threads. Figure 1 shows one of the latter bodies bearing a satellite attached to a nucleolus.

The macrospore mother cell and its nucleus rapidly enlarge. The leptoneuma threads are formed from the chromatic reticulum and

proceed to conjugate (fig. 2). In the synizetic contraction (fig. 3), the chromatic strands usually do not inclose the nucleolus but lie along one side and in contact with it. As only one nucleolus is present at synizesis, it seems likely that fusion of nucleoli has occurred in



FIGS. 1-7.—Macrosporogenesis in *Monarda fistulosa*: fig. 1, nucleus of archesporial cell; fig. 2, nucleus of macrospore mother cell, presynizetic stage; fig. 3, same, synizesis; fig. 4, diakinesis; fig. 5, lateral view of heterotypic equatorial plate; fig. 6, lateral view of heterotypic anaphase; fig. 7, two chalazal macrospores; micropylar cells not yet formed.  $\times 1300$ .

those nuclei which previously contained more than one. As the chromosomes emerge from synizesis, their double nature is evident. After the shortening and thickening of the chromosomes, sixteen pairs can be counted in diakinesis (fig. 4). Size differences exist and the chromosomes may be grouped into eight larger and eight smaller pairs; in each group there is a gradation in size. Two large chromosome pairs bear satellites.

At the equatorial plate stage the chromosomes are smaller and more compact (fig. 5). The behavior of the chromosomes in metaphases and anaphases is regular (fig. 6). Satellite chromosomes can be identified in many of the nuclei of the macrospores (fig. 7).

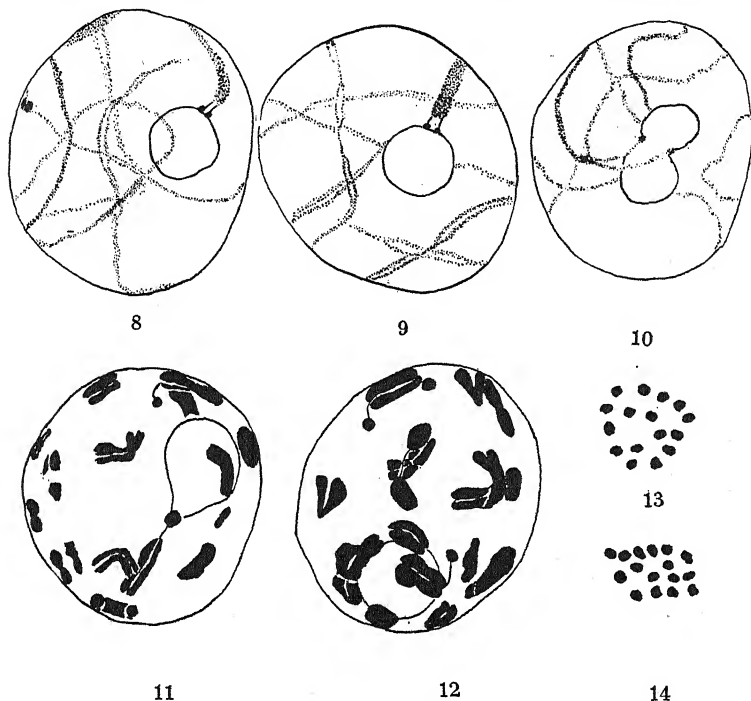
The nucleus of a young microspore mother cell has a chromatic reticulum and one, two, or three nucleoli. When there is only one nucleolus, it is large and probably the result of fusion.

The synizetic knot does not surround the nucleolus which, however, is always in contact with some of the strands. Occasionally a chromatic thread can be seen bearing a pair of satellites that are attached to the nucleolus, but generally the massing of the threads makes it impossible to distinguish such an attachment. When the threads of the synizetic knot become loosened, the loops of the spireme appear double. Small satellites and their fibers at the end of a chromosome can frequently be seen attached to the periphery of the nucleolus (figs. 8, 9). As the chromosomes are loosely paired, both satellites of the pair are distinguishable; they appear to be of about the same size. In some nuclei the nucleolus is constricted; in such a case satellites and their fibers partially encircle the nucleolus at the constricted region (fig. 10). In diakinesis sixteen chromosome pairs can be counted (figs. 11, 12). As in the macrospore mother nuclei, there are eight larger and eight smaller pairs, those in each group differing in size. Two pairs of large chromosomes bear relatively large satellites. One pair of satellite chromosomes is usually in contact with the nucleolus; occasionally this pair lies near the nucleolus but not in contact with it. On the other hand, the satellites of this chromosome pair are not always visible, perhaps having become appressed against the chromosomes. The chromosomes of this pair are most often attached to the peripheral portion of the nucleolus, and in some cases the nucleolus appears drawn out at the point of attachment (fig. 11). The other pair of satellite chromosomes apparently is never attached to the nucleolus.

The bipartite nature of the distal tip of a pair of satellite chromosomes is sometimes visible, but generally the two satellites of the pair lie so close together that they appear as one. In one instance one member of a pair of satellite chromosomes that was free from

the nucleolus has its satellite at one end while the other member has its satellite attached at the opposite end (fig. 12).

At diakinesis some nuclei contain two or three nucleoli which have



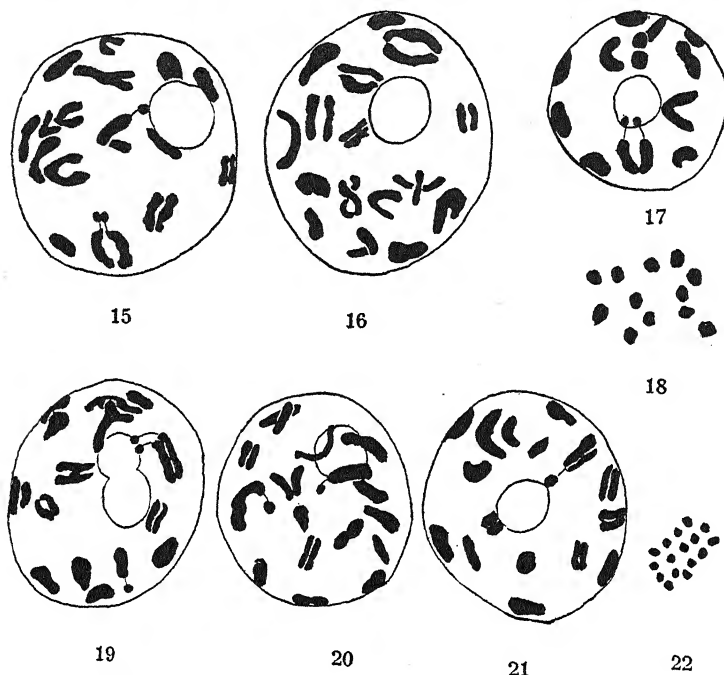
Figs. 8-14.—Meiotic divisions in *Monarda fistulosa*: Figs. 8, 9, open spireme stages showing satellite chromosome pair attached to nucleolus. Fig. 10, open spireme stage, satellite chromosome partially encircling bilobed nucleolus. Fig. 11, diakinesis; sixteen chromosome pairs, one pair of satellite chromosomes attached to nucleolus (which is distorted in shape) and one pair free from the nucleolus. Fig. 12, diakinesis; sixteen chromosome pairs, one pair attached to nucleolus and other pair bearing one satellite at one end, the other satellite at opposite end. Figs. 13, 14, polar views of homoeotypic equatorial plates each showing sixteen chromosomes.  $\times 2830$ .

apparently arisen as a result of budding; other nucleoli at this stage are budding. Such budding has been observed only after synizesis.

Polar views of heterotypic equatorial plates show clearly sixteen pairs of chromosomes. Sixteen split chromosomes are visible in each sister nucleus in interkinesis. In some nuclei at this stage in which

the chromosomes are well distributed, the satellite chromosomes are visible and one pair is seen attached to the nucleolus.

In the homoeotypic division the chromosomes can be counted in



FIGS. 15-22.—Fig. 15, *Monarda didyma*, sixteen chromosome pairs; two pairs of satellite chromosomes, one of which is attached to nucleolus. Fig. 16, *M. didyma*, diakinesis, sixteen chromosome pairs. Fig. 17, *M. punctata*, twelve chromosome pairs, one pair of satellite chromosomes attached to nucleolus. Fig. 18, *M. punctata*, polar view of heterotypic equatorial plate showing twelve pairs of chromosomes. Figs. 19-21, *Nepeta cataria*, diakinesis; sixteen chromosome pairs, one pair of satellite chromosomes attached to nucleolus and one pair not attached. Fig. 22, *N. cataria*, polar view of homoeotypic equatorial plate showing sixteen chromosomes.  $\times 2830$ .

polar views of the equatorial plate (figs. 13, 14). After their formation the four microspore nuclei pass into a resting stage. One, two, or three nucleoli are at first distinguishable, later only one.

#### *Monarda didyma*

Sixteen chromosome pairs are present in the nucleus of the microspore mother cell at diakinesis (figs. 15, 16). The nucleus at this

stage is of about the same size as that of *M. fistulosa*, and in this species also there are two pairs of satellite chromosomes (fig. 15). One pair is attached to the nucleolus; the other is free. Polar views of homoeotypic equatorial plates show sixteen chromosomes, the haploid number.

#### *Monarda punctata*

Twelve chromosome pairs appear at diakinesis (fig. 17). Only one pair of satellite chromosomes is present, the satellites being attached to the nucleolus. The nucleus is smaller than in the other two species of *Monarda*. Lateral and polar views of heterotypic metaphases show twelve chromosome pairs (fig. 18).

#### *Nepeta cataria*

The nucleoli just before synizesis appear bilobed and the lobes are of equal size. Since only one nucleolus is present at synizesis, the nucleoli fuse during the presynizetic stages. In *Monarda*, fusion of nucleoli was thought to occur, but was not observed. In *Nepeta*, however, fusion is apparently a slow process and observable in all nuclei.

In diakinesis, sixteen pairs of chromosomes can be counted (figs. 19-21). Two large chromosome pairs bear satellites; one pair is always attached to the nucleolus. At interkinesis, each of the sister nuclei contains sixteen chromosomes, one of which bears a satellite attached to the nucleolus. Sixteen chromosomes are visible also in polar views of the homoeotypic equatorial plates (fig. 22). The microspore mother nuclei of *N. cataria* are smaller than those of *M. fistulosa* and *M. didyma* although they contain the same number of chromosomes.

#### Summary

1. The haploid chromosome number in *Monarda fistulosa*, *M. didyma*, and *Nepeta cataria* is sixteen, in *M. punctata* twelve.
2. In *M. fistulosa* one pair of satellite chromosomes is commonly in contact with the nucleolus at synizesis, the open spireme stages, diakinesis, and interkinesis. A second pair of satellite chromosomes is free of the nucleolus in diakinesis and interkinesis.
3. Two pairs of satellite chromosomes are present in *M. didyma*

and *N. cataria*. One pair is attached to the nucleolus, the other pair free, at diakinesis and interkinesis.

4. Only one pair of satellite chromosomes was observed in the nuclei of *M. punctata* at diakinesis. It is attached to the nucleolus.

The writer wishes to express appreciation to Dr. C. E. ALLEN for valuable advice and criticism during the progress of the work.

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# CYTOLOGICAL STUDIES IN THE RESEDACEAE

O. J. EIGSTI

(WITH THIRTEEN FIGURES)

## Introduction

The phyletic relationship of species in the Resedaceae has already been established, utilizing floral and morphological features. The present work was undertaken to show how cytological information may be applied to the problems of phylogeny. Apparently no cytological work has been published dealing with the problem of relationship in species of *Reseda*. Accordingly a study was made through chromosome numbers and chromosome morphology, of the species formation and species relationship in this genus.

In the summaries by GAISER (1) on chromosome numbers no report of species in Resedaceae can be found. TISCHLER (6) lists four species, with a reference to a paper by OKSIJUK (4). The chromosome number summaries (table I) include those by OKSIJUK (5), as well as counts by the writer.

## Material and method

The seeds of *Reseda* species were secured from botanical gardens in America and abroad, and the plants used for cytological material were grown at the University of Illinois and at the Carnegie Institution of Washington, Cold Spring Harbor, New York.

Chromosome counts were based on dividing pollen mother cells, and chromosome morphology was studied from dividing root tip cells. The aceto-carmin method was used for pollen mother cell studies. For root tip studies, various fixatives such as those of Allen, Navashin, Lewitsky, and Flemming were used. The material was imbedded in paraffin and sections were cut at approximately 7  $\mu$ . Most of the material was stained with iron-alum haematoxylin.

The counts in all cases were taken from pollen mother cells in the second meiotic metaphase. Chromosome studies in the root tips were made from polar views of the dividing nucleus.



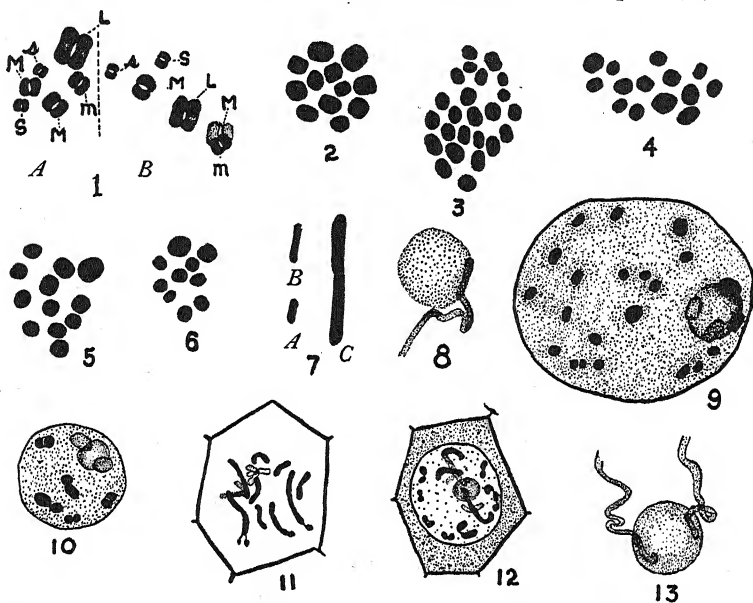
## Results

Figures 1 to 13 show the important features of chromosome morphology in species of the Resedaceae. The chromosomes are very small when compared with those of *Allium* (fig. 7A, C), although there

TABLE I  
CHROMOSOME COUNTS IN THE RESEDACEAE

GENUS	n	2n	INVESTIGATOR
Section Resedastrum Duby			
Reseda			
odorata L.	6	12	4; present paper
inodora Rchb.	6	12	5; present paper
phyteuma L.	6	12	5; present paper
media Lag.	6	.....	5
lanceolata Maire	12	24	Present paper
crystallina Webb	12	24	Present paper
lutea L.	24	48	Present paper; 5
diffusa Ball	24	.....	Present paper
stricta Pers.	24	.....	Present paper
Section Leucoreseda DC.			
alba L.	10	20	4; present paper
Section Luteola			
luteola L.	12	.....	Present paper; 5
luteola L.	13	.....	Present paper
Section Glaucoreseada DC.			
glaucula L.	14	.....	Present paper; 5
glaucula L. (variations)	10	.....	Present paper
glaucula L. (variations)	12	.....	Present paper
virgata L.	14	.....	Present paper
complicata Bory.	14	28	Present paper
complicata Bory. (variations)	15	.....	Present paper
Astrocarpus Neck.	10	.....	5
sesamoides J. Gay.			

is considerable size differentiation (figs. 1, 11, 12). One species studied (fig. 1), with six pairs of chromosomes, diploid number 12, reveals five classes of chromosomes which are as follows: one large pair (*L*), two medium large pairs (*M*), one medium sized pair (*m*), one



FIGS. 1-13.\*—Figs. 1-6, pollen mother cells of: 1, *Reseda odorata* (*L*, *M*, *m*, *S*, *s*, size classes of chromosomes in a species with 6 pairs); 2, *R. crystallina*; 3, *R. lutea*; 4, *R. glauca*; 5, *R. luteola*; 6, *R. alba*. Fig. 7: *A*, *B*, chromosomes of *Resedaceae*; *C*, of *Allium*. Fig. 8, *R. phyteuma*, meiotic prophase nucleolus and attached chromatin strand. Fig. 9, *R. crystallina*, nucleolus and two pairs of prochromosomes. Fig. 10, *R. odorata*, nucleolus and single pair of prochromosomes. Fig. 11, same, polar view of mitotic metaphase. Fig. 12, *R. alba*, prophase in root tip cell. Fig. 13, *R. lanceolata*, two chromatin strands attached to nucleolus in meiotic prophase.

\* All figures drawn with Abbé camera lucida at table level; Zeiss 2 mm. apochromatic objective, N.A. 1.3, and compensating ocular no. 18 used.

medium small pair (*S*), and one small pair (*s*). Studies of the somatic chromosomes (figs. 11, 12) confirm the size differentiations, and additional features such as satellites and primary differentiation (fig. 11), secondary differentiation, and the chromosome-nucleolus association (figs. 8, 9, 10, 12, 13). In all the cases where somatic chromosomes were studied, spindle fiber attachments are median (fig. 11) and thus

produce equal bi-armed chromosomes. The satellites found in a twelve chromosome species occur on the largest pair and may be found dividing before the main body of the chromosome (fig. 11).

Prochromosomes are present in all the species of the Resedaceae studied. One, two, or three pairs of prochromosomes may be attached to the nucleolus during interphasic stages (figs. 9, 10, 12). Chromatin strands in meiotic prophase stages are in contact with the nucleolus (figs. 8, 13).

Chromosome counts 6, 12, and 24, which are haploid numbers, represent a euploid polyploid series in the section Resedastrum of the genus (figs. 1, 2, 3). The other sections in this genus, Glaucorese-da and Leucorese-da, have chromosome numbers of 14 and 10 respectively (figs. 4, 5).

### Discussion

HELLWIG (2) and MUELLER (3) have treated the Resedaceae taxonomically. *Reseda*, which contains about 80 per cent of the total number of species in the family, is divided into four sections. These sections represent morphological, taxonomic, and cytogenetic groups of species. The representatives of each section analyzed cytologically give additional evidence for separation of species into the four groups.

A brief summary of these sections is as follows: Resedastrum, flowers 3 carpeled, stamens numerous (15-40), leaves entire or ternately divided; Luteola, flowers 3 carpeled, apocarpous, stamens yellow, leaves entire; Leucorese-da, flowers 4 carpeled, 5 or 6-merous, stamen number reduced (12-16), leaves pinnate or bipinnate; and Glaucorese-da, flowers 4 carpeled, 6-merous, stamen number (14-22), leaves entire, linear and grass-like in appearance.

The chromosome counts of a species in any given section of *Reseda* are characteristic and representative of the particular section to which they belong. For instance, Resedastrum has eight species with 6, 12, and 24 pairs of chromosomes, or a possible basic number of 6 which characterizes the species in this section. Leucorese-da is represented by *R. alba* with a chromosome complement of 10 pairs; Glaucorese-da with three species studied is characterized by a chromosome complement of 14 pairs. The fourth section Luteola, with

a single species, has a representative and characteristic chromosome complement of 12 pairs. In sections *Glaucoseda* and *Luteola*, variations in the chromosome complement appear which can be explained by meiotic and mitotic abnormalities.

The grouping of species arranged by cytological studies according to chromosome numbers coincides with the grouping of species by their morphological features, and also coincides with grouping of species according to previous taxonomic studies. Cytologically, *Resedastrum* has species with 6, 12, and 24 pairs of chromosomes in their respective complements, and morphologically the species in this section have 3 carpeled ovaries and ternately divided leaves. On the other hand, *Leucoseda*, with a representative species having 10 pairs of chromosomes, has a 4 carpeled ovary and pinnately divided leaves. A third and still different situation is in the section *Glaucoseda* which has three species with 14 pairs of chromosomes. These species have 4 carpeled ovaries, and entire, linear, grass-like leaves. The fourth section, *Luteola*, is morphologically distinct from all other sections by the presence of a long entire leaf and a 3 carpeled apocarpous ovary, and a cytological entity with 12 pairs of small size, uniform chromosomes in the complement.

This coincidence of morphological and taxonomic features with cytological characters represented by chromosome numbers makes it possible to use the chromosome counts as additional information for a key to the species identification in the genus *Reseda*.

A euploid polyploid series is found in the section *Resedastrum* in the following species: *R. odorata*, *R. phyteuma*, *R. inodora*, have 6 pairs of chromosomes; *R. crystallina* and *R. lanceolata* have 12 pairs of chromosomes; and *R. lutea*, *R. stricta*, and *R. diffusa* have 24 pairs of chromosomes. Species formation in this group has undoubtedly occurred through interspecific hybridization and production of fertile plants by duplication of the hybrid complement, as postulated by WINGE (7) in a hypothesis suggested for the production of fertile hybrids.

The evidence for polyploidy in *Reseda* species is derived from the chromosome morphology, prochromosome-nucleolar association, and chromatin strand-nucleolar associations in meiotic prophase. In the first case, species with 6 pairs of chromosomes have five distinct

chromosome size classes and more critical morphological study gives a possible sixth size class. Those species with 12 pairs of chromosomes and 24 pairs of chromosomes in the complement do not have more than the six different size classes such as found in the complement of the 6 paired chromosome species. The species with a complement of 24 chromosomes have fundamentally 4 chromosomes of each of the six size classes, and species with a complement of 48 chromosomes have 8 chromosomes of each of the six respective size classes. Second, the species with 6 pairs of chromosomes show a single large pair of prochromosomes attached to the nucleolus during interphase stage (fig. 10), while those species with 12 pairs of chromosomes have two large pairs of prochromosomes attached to the nucleolus (fig. 9). In the 24 pair chromosome species, three pairs of prochromosomes have been observed attached to the nucleolus. Finally, species with 6 pairs of chromosomes show a single chromatin strand at meiotic prophase attached to the nucleolus (fig. 8), whereas species with 12 pairs of chromosomes have two chromatin strands attached to the nucleolus in meiotic prophase (fig. 13).

The presence of "capsella type" prochromosomes in all species studied suggests an affinity of the Resedaceae with the Cruciferae, in which prochromosomes are known to occur. This phylogenetic relationship proposed on the basis of prochromosomes confirms taxonomic and morphological studies made over a century ago which connected the Resedaceae with the Cruciferae.

### Summary

1. Cytological studies of species of *Reseda* support taxonomic classifications made over a century ago.
2. Species with a multiple number of chromosomes are found in the section Resedastrum. These species have probably originated through duplication of the sets of chromosomes.
3. The basic number of the section Resedastrum is 6.
4. Chromosome numbers 10, 14, and 12 are found to be characteristic for species studied in sections Leucoreseda, Glaucoreseda, and Luteola respectively.
5. The chromosomes of *Reseda* are small when compared with those of *Allium*.

6. Satellites are present on the large chromosomes.
7. The spindle fiber attachments are median and thus produce bi-armed chromosomes which are characteristic for the Resedaceae thus far studied.
8. Five size classes of chromosomes can be distinguished in species with a haploid number of 6 chromosomes.
9. Prochromosomes of the "capsella type" are found in the somatic interphasic stages.
10. The prochromosome association with the nucleolus is not a random association but a specific one in which a particular pair is attached to the nucleolus.

The writer expresses his appreciation to Dr. J. T. BUCHHOLZ for suggesting this investigation and for his criticisms during the progress of the work.

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## HISTOLOGICAL REACTIONS OF BEAN PLANTS TO INDOLEACETIC ACID

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(WITH THIRTY-THREE FIGURES)

### Introduction

For many years problems of tissue differentiation and regeneration have received attention from workers in various fields. More recent activity in the study of some of the developmental phenomena of plants has resulted in the postulation of the existence of hormones which act as stimuli in determining the reactions of tissues as expressed in various environmental ranges. There are now available a rather wide variety of chemically pure compounds isolated from plants or synthesized chemically outside the plant, which are coming to be known commonly as growth substances or plant hormones. AVERY and BURKHOLDER (1) and WENT (13) have recently published reviews and summaries on this general subject.

Among the many substances being experimented with, indoleacetic acid has been shown by ZIMMERMAN and WILCOXON (14) and COOPER (4, 5) to stimulate root development in many plants, and by BROWN and GARDNER (3) to stimulate the production of overgrowths and tumors in the Red Kidney bean. These results, and a long cherished desire to know what histological changes take place in plants when they respond to a growth substance or hormone applied to them, have furnished the reason for beginning these studies.

The bean (*Phaseolus vulgaris*) has been selected as the first subject for detailed examination. In all the experiments here reported, involving some fifteen hundred plants, the variety Red Kidney was used. The plants were grown in pots containing garden soil and kept under average greenhouse conditions. After the seedlings had made

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sufficient growth so that the second internode of the primary axis, which extends just above the pair of heart-shaped leaves to the first compound leaf, had attained a length of 1 to 2 inches and the leaflets of the first compound leaf were beginning to spread out, the stem was cut off squarely about 1 mm. below the base of the petiole of the compound leaf (fig. 1). In some instances axillary shoots were used. All gave approximately the same results. After the shoot was cut

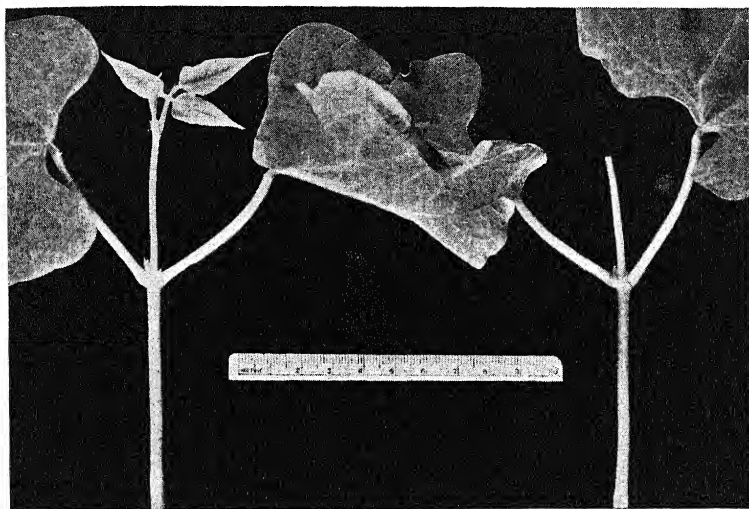


FIG. 1.—Seedlings of Red Kidney bean showing stage of development at beginning of experiments. At left untreated, at right decapitated and film of lanolin mixture applied on cut surface. Reduced.

back, a single application of lanolin containing indoleacetic acid in the proportion of 30 mg. per gram of lanolin was spread over the entire cut surface. Checks treated with pure lanolin and with no treatment other than cutting were also used. No particular attempt was made to regulate precisely the atmospheric moisture or temperature of the greenhouse, to manipulate the light supply, or to vary the soil conditions. What the average gardener would consider good growing conditions were maintained so far as possible. The temperature always ranged above 18° C. Little difference was noted in the character of growth or rate of response made by the plants, whether the experiments were made in the summer or autumn



months. Even so, repetition under a precisely controlled environment would be worth the effort.

Material for histological studies was collected at various intervals after decapitating and treating the stems. Navashin's solution was used throughout as a fixative. The butyl-alcohol paraffin method of imbedding was used and the sections made at 10  $\mu$ .

### Gross responses to treatment

Following decapitation and the application of the lanolin mixture, very little gross response of the stem is noticeable within the first 18 hours; then a faint yellowing of the tissues adjacent to the mixture is evident. By the end of 24 hours the yellowing extends about 0.5 mm. down the stem and the ridges over the principal vascular bundles show a slight swelling just below the cut surface. Within 30 hours the yellowing has increased markedly, especially in the valleys between the ridges. Swelling of the topmost portion proceeds rapidly for at least 1 or 2 mm. down from the cut surface, and by the end of 48 hours the topmost portion of the stem is distinctly flared out. By the end of 72 hours the tip of the stem has attained a diameter nearly twice that of the stem 5 mm. below the cut surface, the whole upper portion is straw yellow, and the tissues in contact with the lanolin mixture are distinctly granular, resembling in appearance a new callus at the tip of a cutting of a root or shoot. The whole end of the stem continues to enlarge, as a rounded dome-shaped mass, and by the end of 110 to 120 hours the glistening tips of root primordia are evident about the whole periphery of the tumor adjacent to the line at which the original cut was made. Within 144 to 168 hours this crown of adventitious roots becomes more evident and some of them emerge beyond the surface of the tumor (figs. 2, 26). If the atmosphere is very moist these roots elongate rapidly and, if favorable conditions are maintained, may continue to grow indefinitely. If the atmosphere is somewhat dry, many of the roots may not emerge beyond the superficial tissues of the tumor, although they persist without further elongation for an indefinite period. If the atmosphere about them becomes saturated with moisture or if they are brought into contact with some moist medium, they may become active and elongate.

Even though the atmosphere remains dry, the tumor as a whole does not cease growth. Although the periphery of the tumor from 1 to 2 mm. from the edge, close to the base of the adventitious roots,

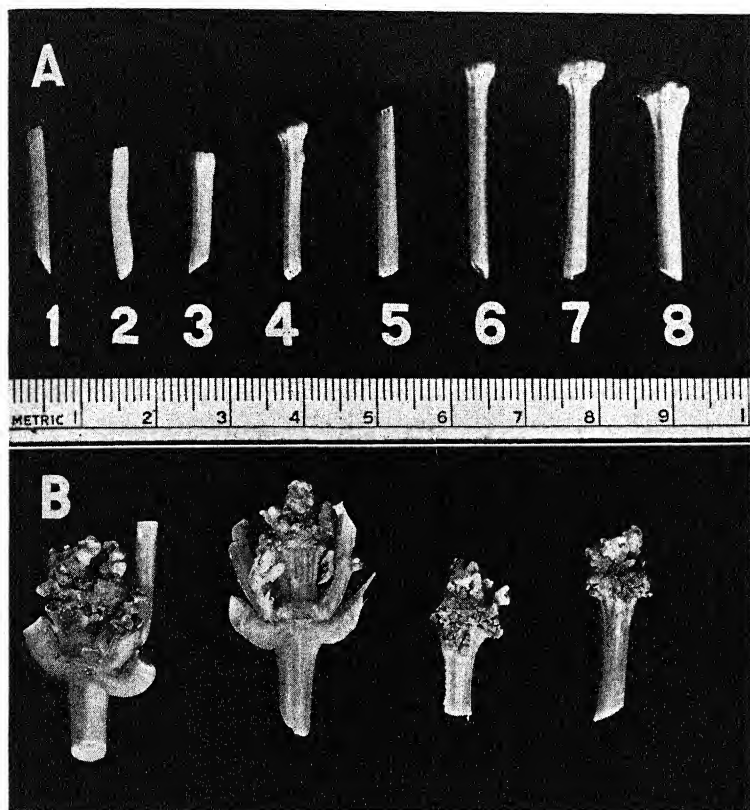


FIG. 2.—Stages in tumor development (time given in hours after treatment) *A*: 1, just after decapitation; 2, 30 hours; 3, 48 hours; 4, 66 hours; 5, 72 hours not treated; 6, 72 hours treated; 7, 144 hours; 8, 168 hours. *B*: four apical tumors 28 days after decapitation and treatment of stem. Adventitious roots have not emerged, but prominent ridges and rows of small tumors over the vascular bundles are evident, as is also the central apical mass of proliferating tissue. Natural size.

may remain inactive, masses of tissue in the form of cones, balls, and other irregular and fantastic shapes grow upward and outward from the central portion, overtopping the crown of roots (fig. 2*B*). The tumors eventually may become 4 or 5 cm. in diameter. They

are gnarled and irregular in contour, greenish gray, with scurfy, gray patches scattered irregularly over their surfaces.

In general the stems which are cut back and treated with pure lanolin continue to grow slightly in diameter, remain dark green, and after a variable number of days develop a phellogen slightly below the plane of the cut (fig. 30). If neither lanolin nor the lanolin mixture is applied to the cut surface, the tip of the stem may dry

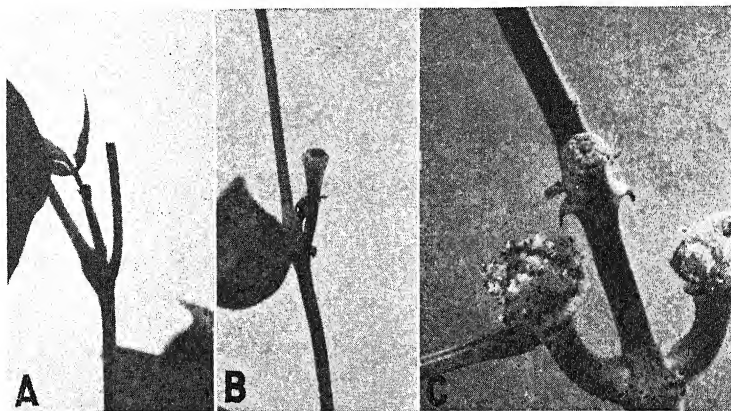


FIG. 3.—*A*: axillary branch 4 days after decapitation, no treatment. *B*: similar branch 4 days after treatment with lanolin mixture. Reduced. *C*: three branches 20 days after decapitation; upper center not treated but covered with callus, the other two treated at time of cutting with lanolin mixture. About natural size.

out, shrivel, and die back for a varying distance from the cut; or callus formation may begin near the periphery of the stem and continue until the entire end has been covered over by a dense, firm, somewhat wrinkled and superficially suberized mass of tissue (figs. 3C, 32). This callus is largely derived as a proliferation of the phloem (figs. 32, 33).

The tissues of the veins and petioles of the leaves, developing pods, and other parts of the plant readily proliferate and form tumors when the lanolin mixture is applied, whether or not the epidermal tissues are cut away or ruptured before such application is made (figs. 4, 5).

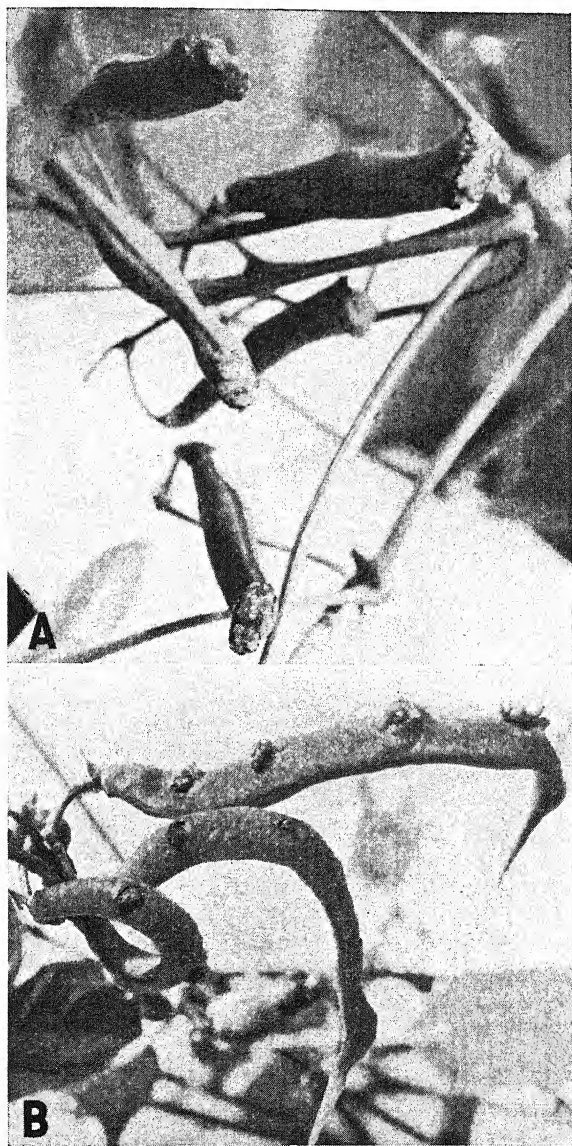


FIG. 4.—*A*: bean pods 16 days after being severed in half and cut surface treated with lanolin mixture. *B*: similar pods 28 days after being punctured with needle and treated at puncture with lanolin mixture. Slightly reduced.

### Histological details

DOUPEL (6) has described the normal anatomical details of the Black Valentine bean. The structure of the stem of the Red Kidney variety closely parallels her description. A transection of the second internode of the main axis taken at the level at which the plants were decapitated is shown in figure 6. It is obvious that most of the cells have not reached maturity, although the several main regions

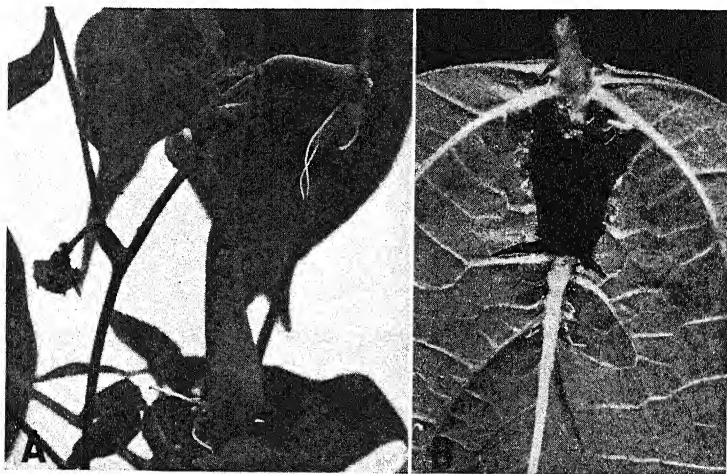


FIG. 5.—*A*: roots developed from end of bean pod 13 days after being severed and cut surface treated. Reduced. *B*: roots formed on midrib of bean leaf 18 days after severance of vein and local treatment with lanolin mixture. About natural size.

of the stem are well defined. The epidermal and cortical tissues, including the endodermis, have moderately dense cytoplasm and prominent nuclei. Many of the outer cortical cells have chloroplasts. The pericyclic cells over the primary phloem show the beginning of thickening to form fibers, but still have dense cytoplasmic contents and apparently nuclei which have not disintegrated. All the cells of the primary phloem are embryonic except perhaps the sieve tubes. The cambium is active; the zone of undifferentiated tissue is not more than four or five cells wide. The cells composing the medullary rays as well as those of the pith have dense contents but show no marked meristematic activity.

Eighteen hours after the application of the indoleacetic-lanolin mixture some definite changes are observable. The cells of the first one or two layers just below the treated surface are frequently killed, but beneath these the following changes are observable. The chloroplasts in the peripheral cells of the cortex have disappeared. The cortical cells have undergone marked enlargement; their cytoplasmic content appears much denser; and a few cells, especially those adjacent to the endodermis, show tangential divisions. The endodermal cells have enlarged markedly, especially in the radial dimension; their content is denser and starch grains are no longer readily demonstrable with the iodine test. The pericycle cells have also increased in size, but their walls show very slight thickening. The parenchymatous cells of the primary phloem appear meristematic, as do those of the secondary phloem; the zone of undifferentiated cells adjacent to the cambium is much wider, owing to an increased number of cells and the larger size of many of them.

Subsequent developments, beginning with material which has been treated for 30 hours, are indicated in the photomicrographs. In some of the experiments specimens were taken at 6-hour intervals from the beginning up to a total duration of 168 hours. Only those examples which show significant progressive developmental changes are presented here. Not every stem used in each experiment responded at exactly the same rate, the more highly vegetative plants seemingly advancing more rapidly than those less vegetative; but in general any particular lot of plants subjected to the same type of treatment showed a high degree of uniformity. There were some marked differences in response of the plant as a whole and of the tissues in the vicinity of the application of the lanolin mixture, depending upon whether the epidermis was or was not injured, whether the applications were made on one side of the stem or completely encircled it, whether the application was in a thick or a thin layer, and other variations. We shall endeavor to present at a future time the details of these varied experiments, the histological pattern of tumors older than 168 hours and of the plants on which they are borne. The present discussion deals mainly with material not older than 168 hours.

One of the most obvious results of the application of the lanolin

mixture to the stem tissues of the bean is the marked stimulation to meristematic activity of some of the tissues and not others. This is first characterized by increased nuclear division. Some cells of the endodermis may have as many as six nuclei before any visible walls are formed, while in some of the larger cells of the pith as many as twelve or even more nuclei may be found.

In none of our experiments has the epidermis shown marked increase in meristematic activity. The cells may enlarge more rapidly than those of untreated stems. There is a slight increase in the number of radial divisions, but the cells do not divide tangentially. When the concentration of 30 mg. of indoleacetic acid to 1 gram of lanolin is used, the epidermal cells in immediate contact with the mixture are likely to be killed, but otherwise they persist for an indefinite period unless the expansion of tissues within ruptures the surface, or the cortical cells beneath them die.

The parenchymatous cells of the cortex show varying degrees of response. Those next to the epidermis enlarge, often very greatly, but may show no change other than an increasing density of the cytoplasm and the disappearance of the chloroplasts and any included starch grains. Those near the endodermis generally become highly meristematic, their nuclei undergoing rapid divisions, often with subsequent wall formation delayed, so that the cells are multinucleate. After 110 to 120 hours meristematic activity is greatly decreased near the cut surface; the outermost cells mature, die, and become disrupted by the expanding roots and other tissues formed centripetally to them. The cells which are 3 or 4 mm. distant from the cut surface are not so highly meristematic at any time as are those near it, and they frequently do little more than enlarge. They apparently remain alive for a considerable period, although as time goes on the chlorophyll disappears from them at distances of more than 5 cm. from the developing apical tumor.

Of all the cortical tissues, the endodermis is most responsive. Within 24 hours the cells near the treated cut surface enlarge greatly, starch disappears from them, their cytoplasmic contents become dense, and soon thereafter rapid nuclear divisions begin. These original cells generally elongate markedly in a radial direction. Sub-

sequently tangential divisions occur, and the original single layered endodermis becomes a wide meristematic band consisting of cells which are often indistinguishable from those of the adjacent cortical parenchyma. In the vicinity of the apical tumor this band of cells remains active for a long period and some of the cells composing it may mature as tracheids with large pits, others as parenchymatous cells with large vacuoles, while many form sheath-like coverings over the developing roots.

The behavior of the endodermal cells at distances of 2 mm. to as much as 3 cm. from the apical tumor, and hence well away from any direct contact with the indoleacetic acid except as it finally diffuses to them through other tissues, is of special importance. The reactivity and multiplication of longitudinal columns of these cells so closely parallel the tumor strands of crown gall that they may well be considered as being analogous to and perhaps homologous with them. Similar strands are developed in other tissues also, as described later. As indicated in figure 17, within 72 hours endodermal cells distant at least 1 cm. from the treated surface show marked radial enlargement and frequently tangential divisions as well. They do not, however, become so highly meristematic as those close to the developing apical tumor; but the derivatives undergo division in all planes, and form, especially over the primary phloem of the vascular bundles, groups of dividing cells concentrically arranged. The cells at the center of such a group develop into xylem elements; those nearer the periphery continue division and differentiation, so that in any given section it is possible to see at the end of 168 hours vascular strands with a central mass of xylem surrounded by dividing cells and phloem elements, the whole having been derived from cells of the original endodermal layer. At this time too, cells of the endodermis as far away from the cut surface as 3 cm. show the same type of activity as did those nearer it at a shorter time interval. Thus it is possible to observe within a 4 cm. length of stem all stages in development of these endodermal vascular strands, beginning with the first regions of activity at the lower level to large conspicuous strands with xylem elements at the center near the apical tumor (figs. 22, 23). After some days there



may be so great an enlargement of these and other strands developed in other tissues that the cortex is ruptured and small corrugated tumors appear along the stem.

Some specimens show root primordia originating in the outer layers of such active endodermal cells, accompanied by the differentiation of vascular structures through the intervening tissues back to the xylem of the original bundles in the stem. These are no doubt the beginnings of roots which later emerge from the stem at varying distances below the apical tumor.

The cells of the pericycle which would normally mature as fiber cells do not appear to become markedly meristematic in response to applications of the lanolin mixture. If they are relatively embryonic and lie adjacent to the treated surface they do enlarge and may undergo several divisions, but they do not elongate greatly nor do their walls become much thickened (fig. 18). They are most frequently crushed or pushed from their original position by the tissues located centripetally to them. Two or 3 mm. distant from the cut surface the walls of the pericyclic cells continue to thicken and the fibers mature much as they do in an untreated stem. The cells over the ray remain thin walled and undergo a few divisions, but often are not distinguishable as a tissue from the exceedingly active endodermal cells lying outside them or those of the ray nearest them.

The primary phloem is highly sensitive in its response to the stimulus of the indoleacetic acid, and near the surface of application the parenchymatous cells of this tissue proliferate abundantly and frequently elongate radially. The new cells thus formed may for a time be multinucleate, but later walls form in all directions, especially in the tangential plane. Eventually the cells enlarge and many of them mature as tracheids with large simple pits. Others remain parenchymatous and ultimately die, or become ruptured and torn by the expansion of other tissues near them. At greater distances from the tip the primary phloem cells undergo the same types of change and development as do those of the endodermis already detailed. Thus after 168 hours, strands of actively dividing cells located within the area of the primary phloem extend 2 or 3 cm. down the stem. Later these develop xylem elements at their

centers and may extend for much longer distances. Finally radial expansion and development result in masses of cells which rupture the tissues exterior to them and protrude as tumors and root initials of varying size in more or less parallel or longitudinal rows along the stem and downward from the large apical tumor.

The parenchymatous cells of the secondary phloem are as sensitive as are those of the primary, and are among the first tissues to show a decided response after the application of the lanolin mixture. Adjacent to the treated surface, meristematic activity of these cells is very great. The cells enlarge in all directions, particularly radially, thus pushing outward all tissues exterior to them and forcing the sieve-tube companion-cell groups wide apart and in all directions. This proliferated phloem tissue forms a large part of the developing apical tumor in its early stages and enters into the lateral portions of the adventitious roots which are eventually formed at the top of the stem. It is doubtful whether they contribute to the apical meristem of such roots when their histogens are eventually differentiated. At increasing distances down the stem away from the treated surface the parenchymatous cells are decreasingly active, and show far less response in such regions than do the cells of the endodermis, primary phloem, or the pith.

With the exception of the endodermis, the cambium is probably the most sensitive in its response to this type of stimulation. Its cells proliferate rapidly, but differentiation of the derivatives is much delayed. Thus a very wide band of highly meristematic cells comes to exist between those elements of the secondary xylem and phloem which were differentiated before the lanolin mixture was applied. Later large pitted tracheids mature more or less at random throughout this tissue and some of the cells near the phloem mature as phloem elements. The response of the cambial cells may be manifested several millimeters below the surface of application, but not at distances so great as in the case of the endodermis or the phloem, especially the primary phloem.

In point of time the cells of the pith are relatively slow to respond, but when meristematic activity is initiated the cells proliferate rapidly and their activity continues over a longer period of time than does that of the cortex (excepting the endodermis) or of the second-

ary phloem. Activity is first manifested by those cells which lie adjacent to the metaxylem vessels and the elements of the protoxylem. At first wall formation follows nuclear division very closely, but later many of the cells may be multinucleate, wall formation taking place subsequently. Activity of these parenchymatous cells often forces the elements of the xylem apart and their linear arrangement becomes greatly confused. Activity is begun somewhat later by the larger cells of the pith, but eventually practically all the cells differentiated before the lanolin mixture was applied become highly active. Throughout the mass of resultant tissue there are enlarged parenchyma cells, strands, zones, and islands of meristematic cells, tracheids, and phloem elements in every possible relationship. It is this conglomeration of tissues which continues to develop over a very long period of time and eventually makes up a large part of the apical tumor which assumes varied and grotesque shapes, as mentioned previously.

The cells of the ray parenchyma which lie between the points of the xylem respond almost precisely as do those of the pith, except that larger numbers of them mature more rapidly as pitted tracheids. Many of the cells remain meristematic for a long time and some of the derivatives of these also mature as tracheids and as clusters of sieve tubes. The cells of the ray which lie on a line with those of the phloem behave much as do those of the phloem parenchyma. Meristematic activity is very great, and as such activity continues and the derived cells nearer the center of the stem enlarge, a dense meristematic mass, flanked at either side by the highly active phloem parenchyma, is pushed outward toward the periphery of the stem. In some specimens this mass occupies a comparatively narrow wedge between the phloem groups; in others it is much wider. Often it is difficult to define exactly, but its limits can generally be decided from the patches of sieve tubes which lie at either side. In stages of development between the ages of 66 and 80 hours, masses of cells with denser content can be seen just within the band of endodermal cells; and after 80 hours the outlines of adventitious roots are readily discernible, made up partially of ray cells and partially of cells of the phloem.

The development of a circle of adventitious roots within a dis-

tance of 1 or 2 mm. from the cut surface of the stem is one of the most striking characteristics of the bean stem when such surface is treated with indoleacetic acid in lanolin. The general histological responses of the several tissues have just been described. In the genesis of the roots several tissues are involved. It seems doubtful whether the pericycle plays much part. Over the rays there are two to four thin walled cells which undergo some divisions, but such divisions are not extensive either radially or tangentially. The cells of the ray immediately inside these, however, proliferate considerably. So rapid and so extensive are these divisions, that if histogens for the adventitious roots are developed by the ray cells in the early stages of tumor formation they are not clearly indicated. In fact, such a histogen is difficult to distinguish even in stages 110 hours old, although groups of more highly active cells occupying the position of the ray between two adjacent masses of phloem and even within the phloem areas are readily discernible in 48 hours (fig. 8). In stages of development older than 80 hours the outlines of the roots become well defined, owing to the enlargement and increasing vacuolization of the cells near the center of the phloem masses of each of the vascular bundles, over the xylem, and beneath the pericycle. The lateral cells of two adjacent phloem groups remain meristematic for a considerable period and serve to make up the bulk of the outer portion of any given root. The central core of each root is derived largely from ray cells, and its apical histogen seems to be derived from the same source. At least when it is possible to be certain of the apical histogen, the cells which compose it lie nearer the center of the stem than do those of the proliferated endodermis, and probably also those of the pericycle, although this is much more difficult to determine. Following this general blocking out of the root, xylem elements are differentiated near the base and periphery of the inner core of cells; many mature as large pitted tracheids abutting the tracheids already differentiated from those ray cells which previously lay between the xylem vessels of two adjacent bundles. Those nearer the center of the core elongate greatly and differentiate mainly as tracheids, although others appear as tracheal segments. Phloem elements mature in the outer portions, especially in contact with the sieve tubes and other cells

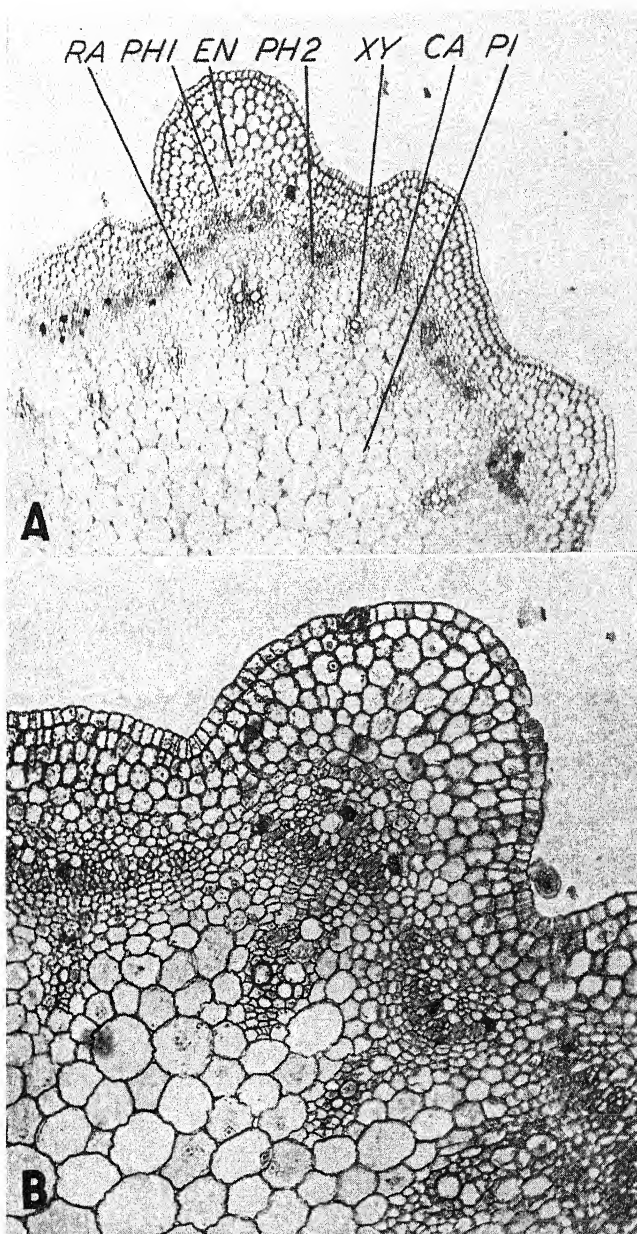


FIG. 6.—*A*: transection of top of second internode of bean at time of initial treatments, showing various tissues and stage of development (*ra*, ray; *ph*<sub>1</sub>, primary phloem; *en*, endodermis; *ph*<sub>2</sub>, secondary phloem; *xy*, xylem; *ca*, cambium; *pi*, pith). *B*: sector of same enlarged; pericyclic fibers over the smaller bundles are more nearly mature than those over the larger.

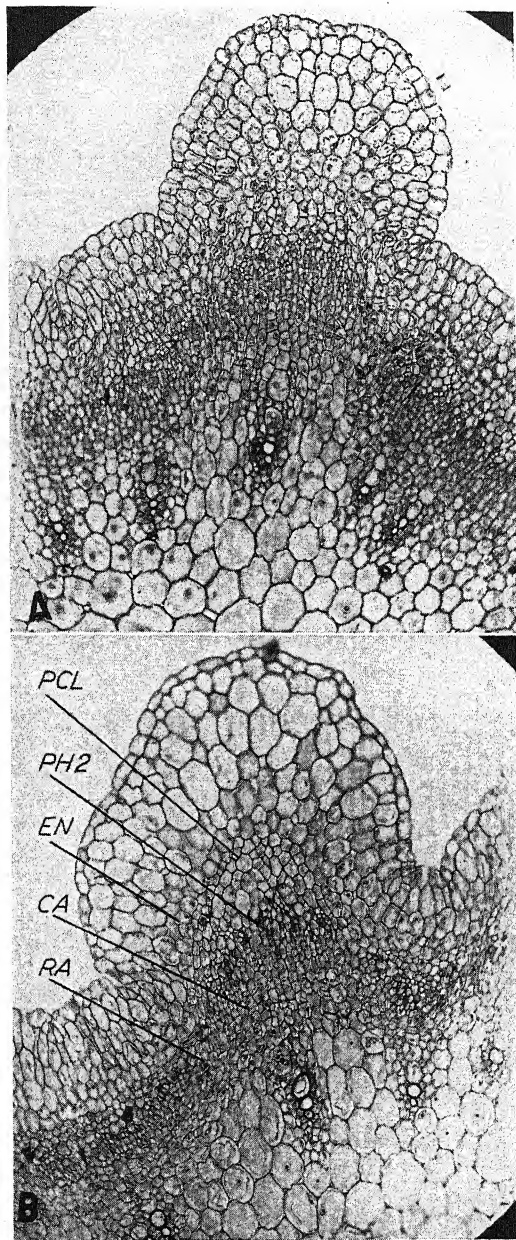


FIG. 7.—Thirty hours after treatment. *A*: section about  $150\ \mu$  below treated surface. Pericyclic cells are embryonic, primary and secondary phloem parenchyma in stems show increase in meristematic activity, derivatives from the cambium remain meristematic and continue division, ray parenchyma is active, cytoplasm of the pith cells is dense. *B*: about 1.5 mm. farther down the stem. Pericyclic cells show increased maturity, other tissues about as in *A*.

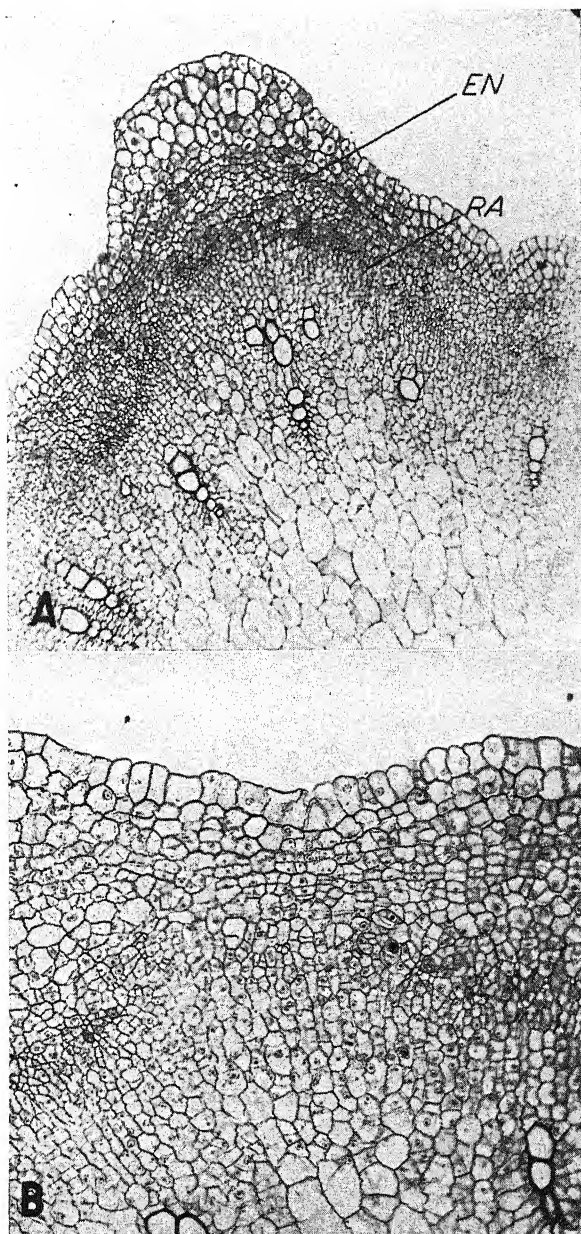


FIG. 8.—Forty-eight hours after treatment. *A*: section about 600  $\mu$  below treated surface. There is marked increase of meristematic activity of all tissues except the pericycle and epidermis. Some xylem vessels are pushed apart by active ray and xylem parenchyma cells. Pith adjacent to protoxylem and metaxylem is meristematic. There is slight differentiation of secondary tissues from the cambium. *B*: same enlarged, showing tangential division of cortical parenchyma and endodermis, also active ray cells.



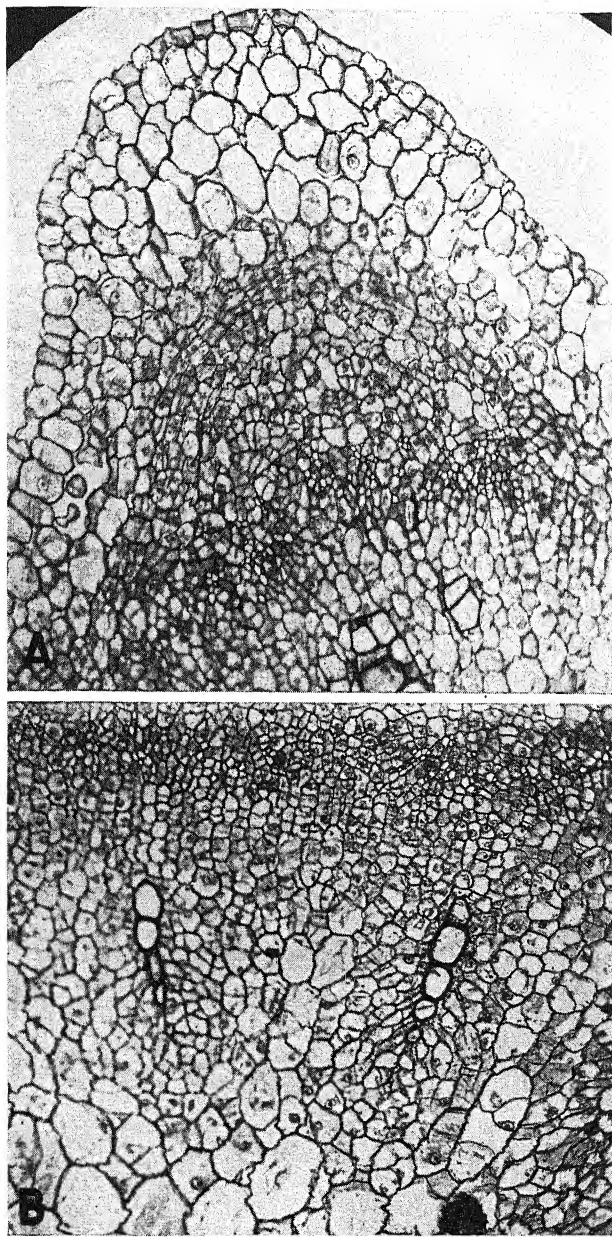


FIG. 9.—*A*: same stem as fig. 8; section about 1.5 mm. below fig. 8. All tissues except the endodermis are less active. Secondary elements from the cambium are fewer and more nearly mature. *B*: same as fig. 8 *B*, showing activity of pith and ray cells.



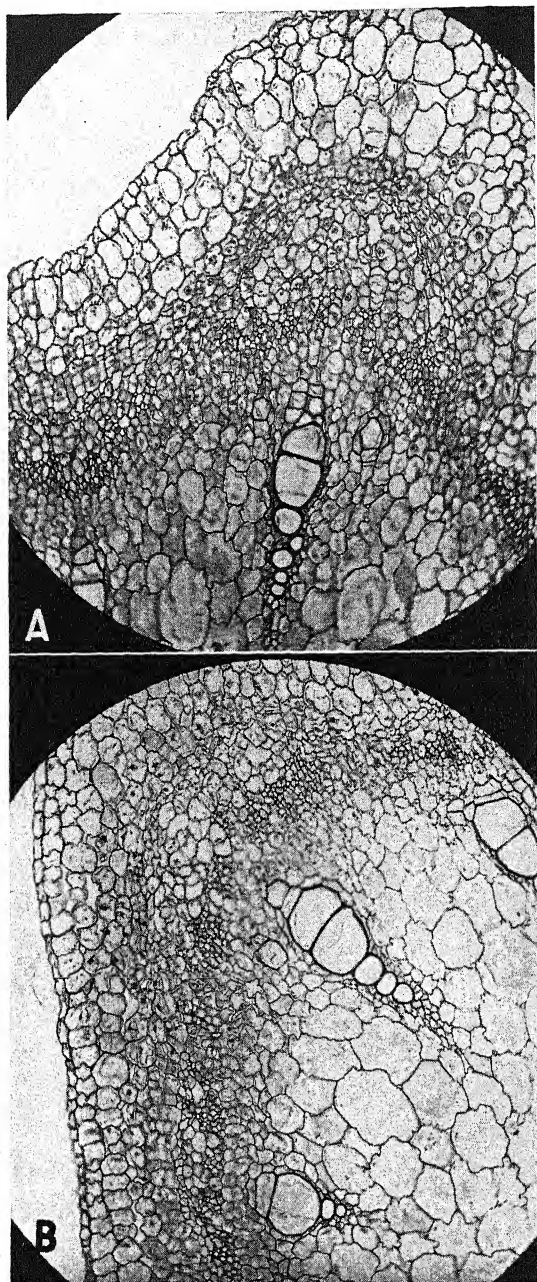


FIG. 10.—Same stem as figs. 8 and 9. *A*: section about 2 mm. below treated surface. Endodermis and primary phloem parenchyma are meristematic, the other tissues much more nearly mature. Cambial zone is narrow with few meristematic secondary derivatives. *B*: section 4 mm. below treated surface. With the exception of the cambium and endodermis, all tissues are nearing maturity. Endodermal cells, especially those over the smaller bundles, have undergone several divisions in tangential plane.

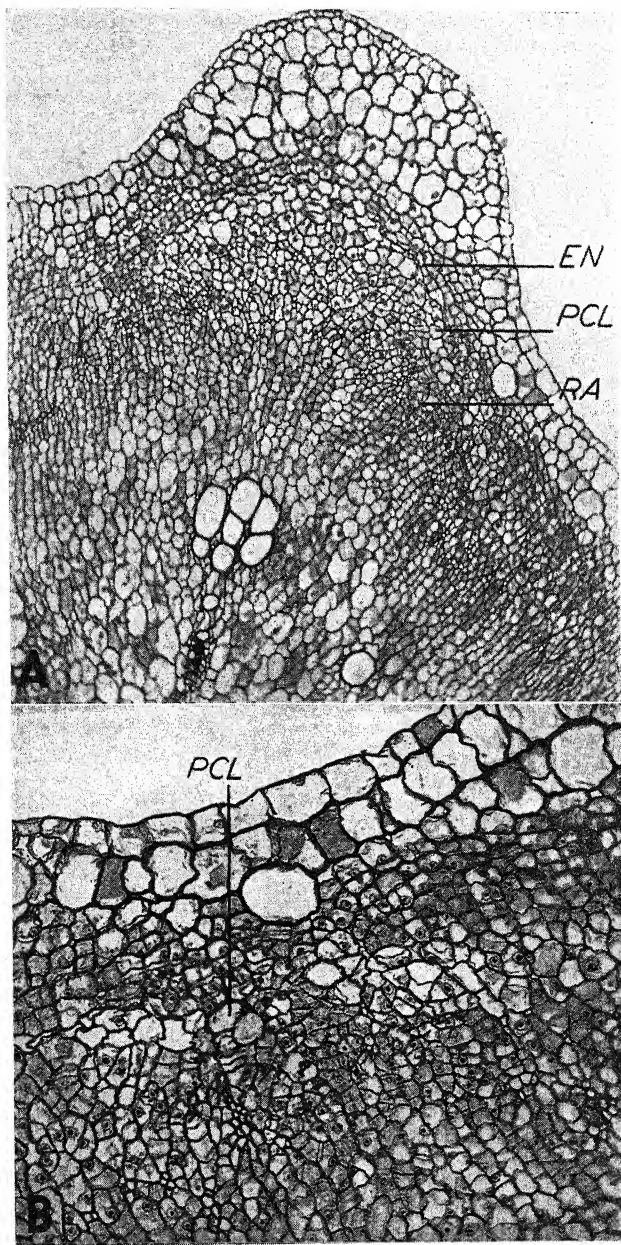


FIG. 11.—Sixty-six hours after treatment. *A*: section about 1 mm. below treated surface. Increased activity of all tissues except the pericycle as compared with 48-hour material. Pericyclic cells are comparatively much larger and their walls thicker; ray cells have proliferated considerably and the whole mass has expanded radially, paralleling the expanding phloem regions and derivatives of the cambial zone. *B*: enlarged view of ray in *A*. Pericyclic cells over it, on a line with those maturing over the bundles, show slight activity although the ray cells are much more active.

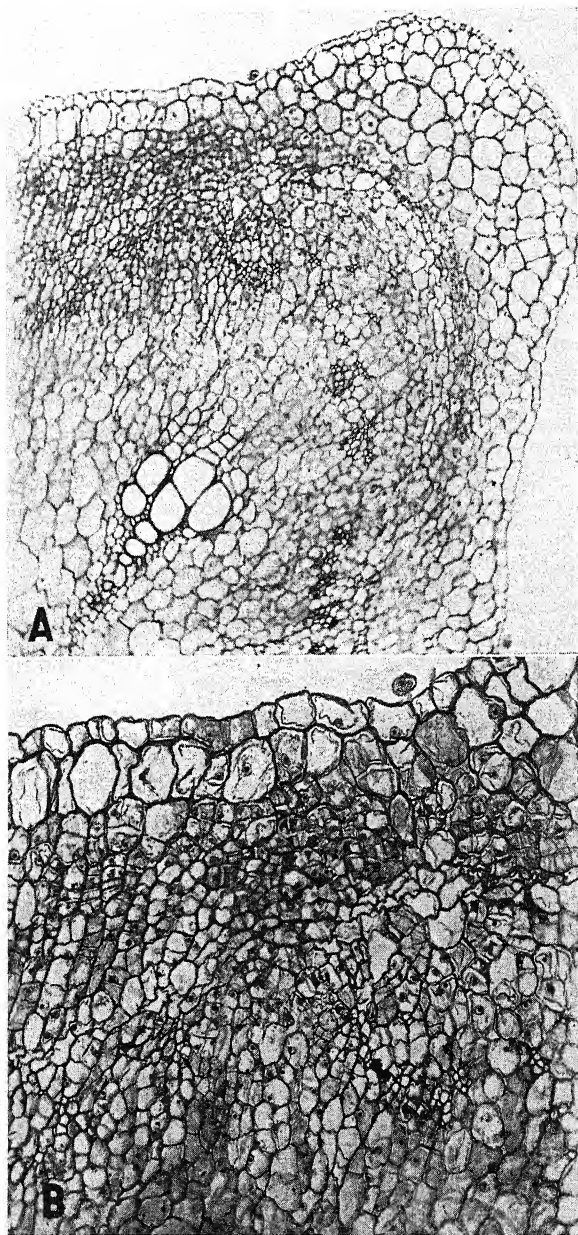


FIG. 12.—Same stem as fig. 11. *A*: section 5 mm. below treated surface. Less activity than in fig. 11, but because of longer time interval after treatment the tissues show greater meristematic activity than do those in fig. 10 *B*. *B*: ray and adjacent tissues enlarged; pericyclic cells over the bundles approaching maturity; endodermal derivatives and inner cortical parenchyma very active.

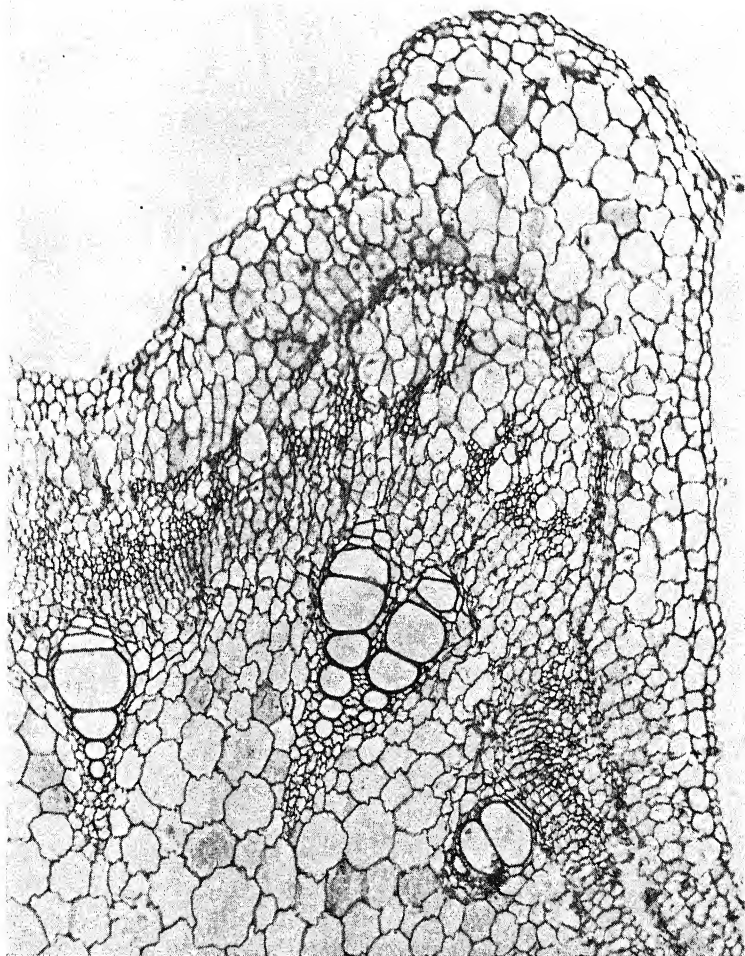


FIG. 13.—Same stem as fig. 11, section 9 mm. below treated surface. Endodermis, primary phloem of larger bundles, and pith near the protoxylem and metaxylem show some response; other tissues except the cambium nearing maturity. Rays show virtually no response. Slightly below this level no obvious response to the treatment (*cf.* fig. 17).

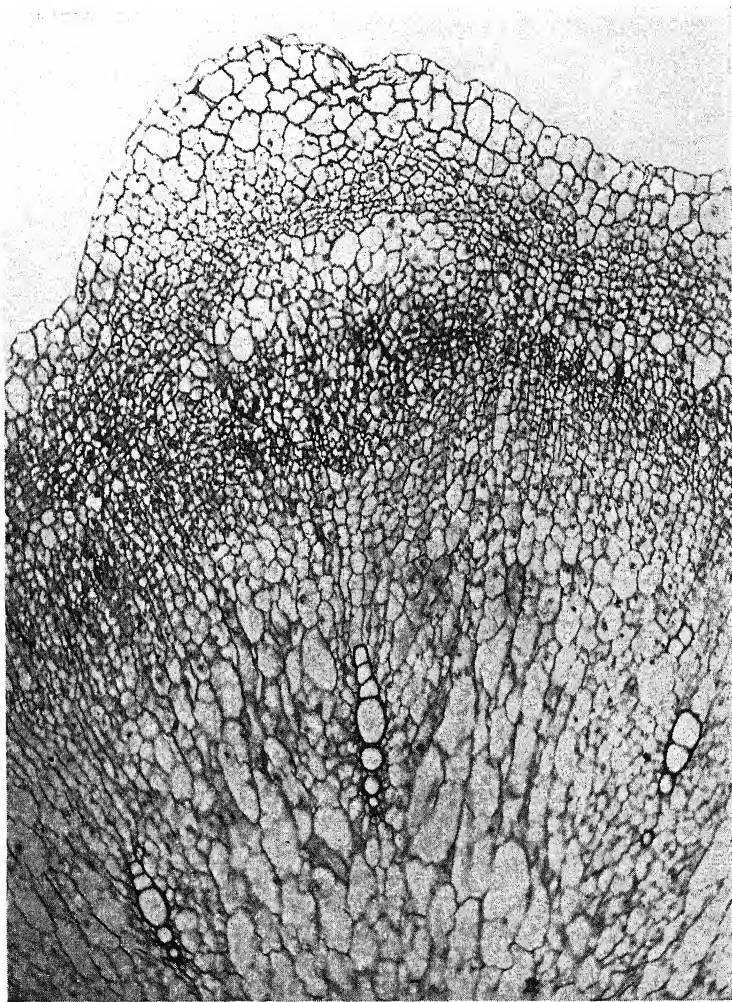


FIG. 14.—Seventy-two hours after treatment, section about 1 mm. below treated surface. Pericyclic cells much enlarged and nearing maturity although walls have not thickened greatly. Endodermal derivatives constitute a wide meristematic zone. Phloem parenchyma and derivatives from the cambium remain meristematic. Ray cells just within the pericycle constitute a meristematic mass distinguishable with some difficulty from the flanking phloem cells. The cells nearer the xylem vessels and between the bundles are meristematic and have elongated markedly in radial direction, thus pushing the outer cells of the ray toward the periphery of the stem.



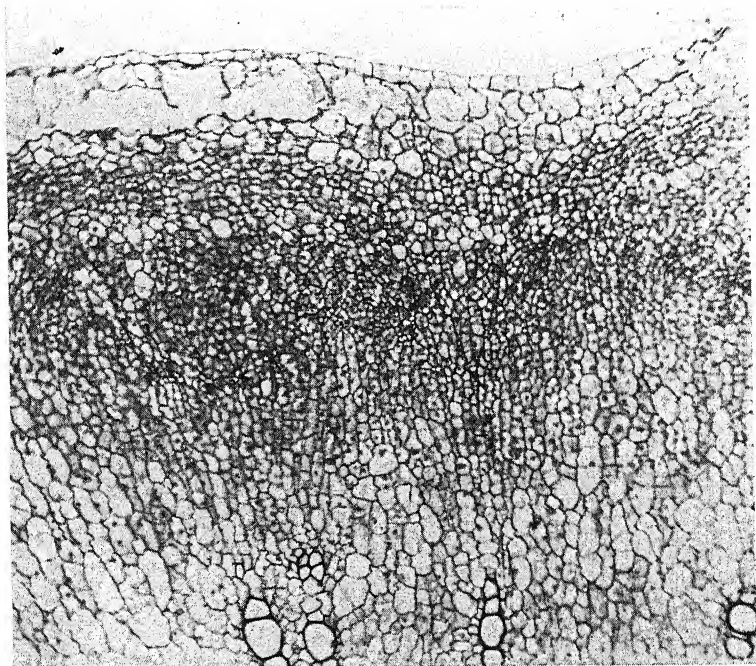


FIG. 15.—Same as fig. 14. Outer cortical cells disrupted by expansion of tissues within. The section lies in plane in which adventitious roots are formed. The outlines of such a root are shown in ray near center of illustration. At this stage it is very difficult to distinguish definite histogens for the new roots, if such histogens are present.

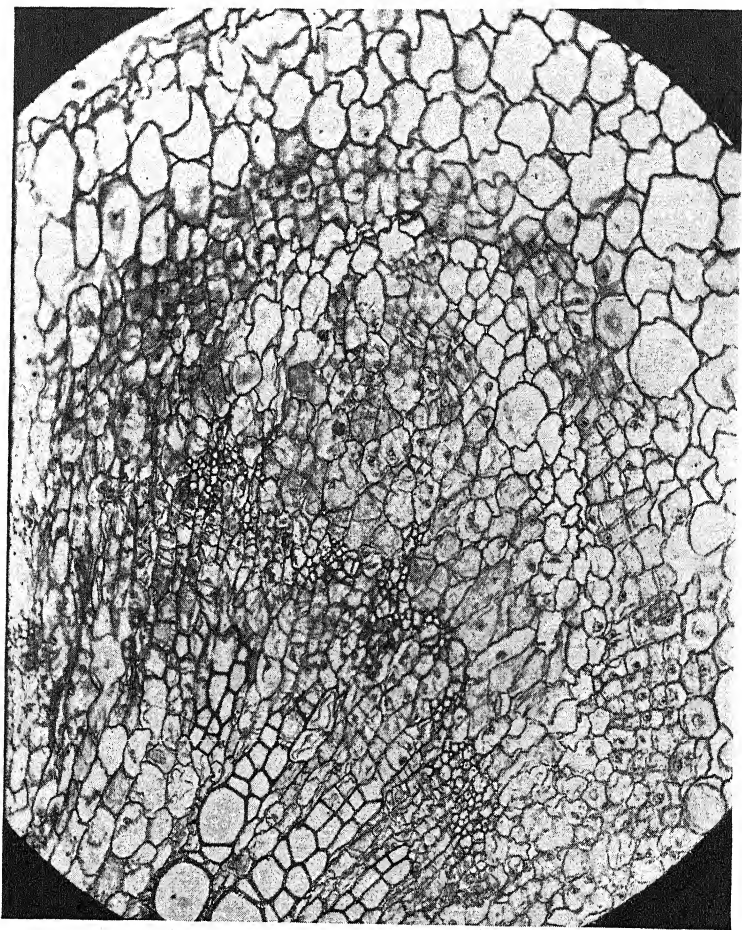


FIG. 16.—Same stem as fig. 14, section about 2.5 mm. below treated surface. Cambial derivatives have matured close to the cambium; pericycle cells nearly mature; endodermis proliferated. Within the region of primary phloem there is great meristematic activity and near the center is the beginning of a vascular strand. Such a strand at a somewhat more advanced stage is shown in fig. 21. Such strands eventually extend continuously for distances of several centimeters in the stem, the more nearly mature portions at the top and the younger progressively down the stem.

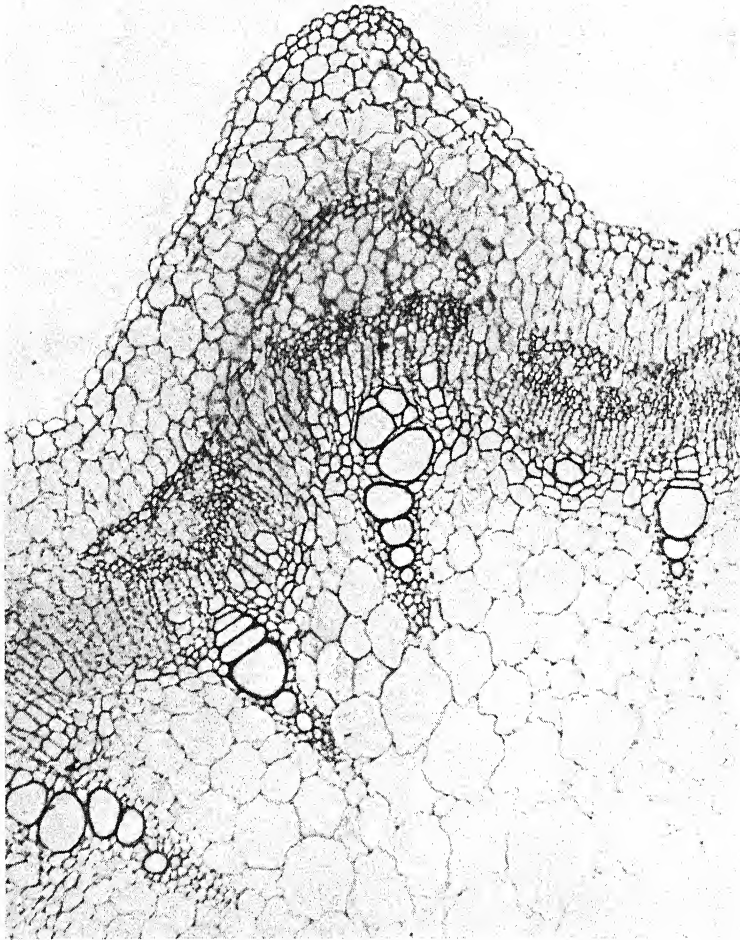


FIG. 17.—Same stem as figs. 14 and 16, section 1.2 cm. below treated surface (*cf.* fig. 13). Response is shown by cells farther away from surface of application and there are more tangential divisions of the endodermal cells. Sections of this stem taken 1.7 cm. from the treated surface showed the endodermal cells greatly elongated in a radial direction and tangential divisions of some of them. Some ray cells have matured as tracheids. The latter are abundant in the secondary xylem. The fibers over the primary phloem are not yet fully mature but show no divisions.



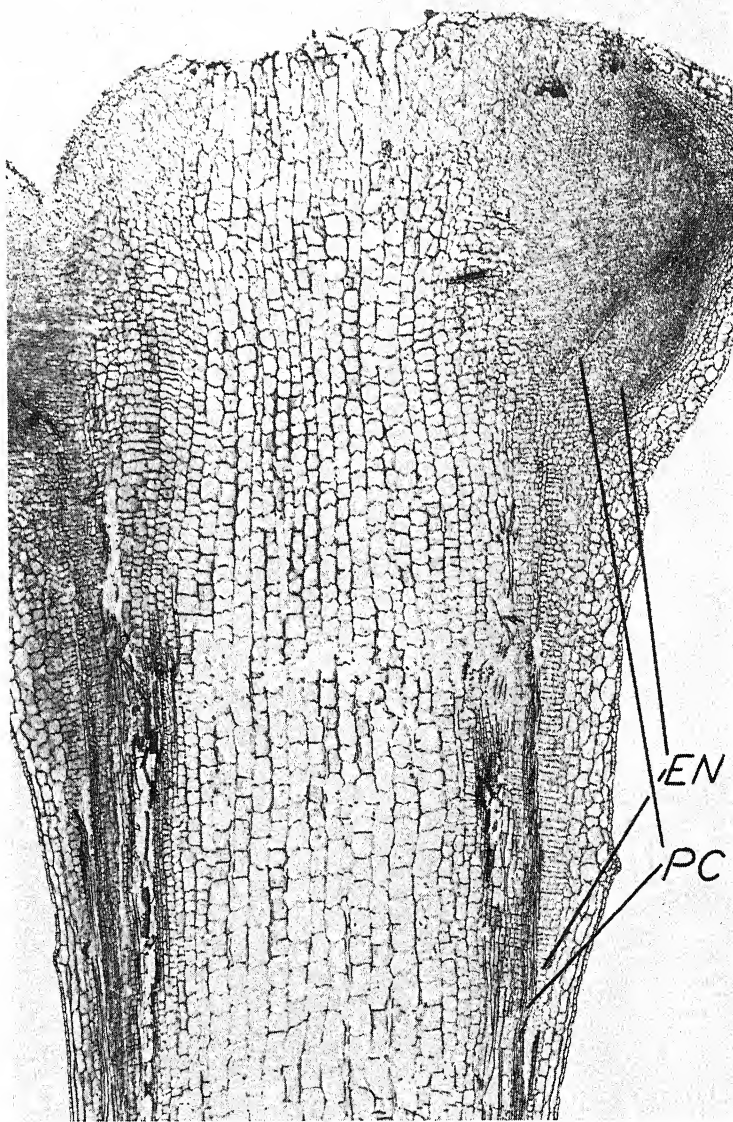


FIG. 18.—Ninety hours after treatment, median longitudinal section. Lower portion of adventitious root, nearer pith, is through a ray; distal portion is not strictly median. Pericycle cells (*pc*) have matured as fibers in lower portions of stem, but in upper portions nearer the surface of application they have divided and enlarged as parenchymatous elements. The tip of the root lies just beneath this layer. Endodermal cells (*en*) have responded to stimulation for a long distance down the stem. Older stages would show such response at even greater distances. Proliferated phloem extends along margins of the adventitious root whose central portion is made up largely of proliferated ray cells. The pith is active, particularly near the top. Cortical parenchyma is slightly active.

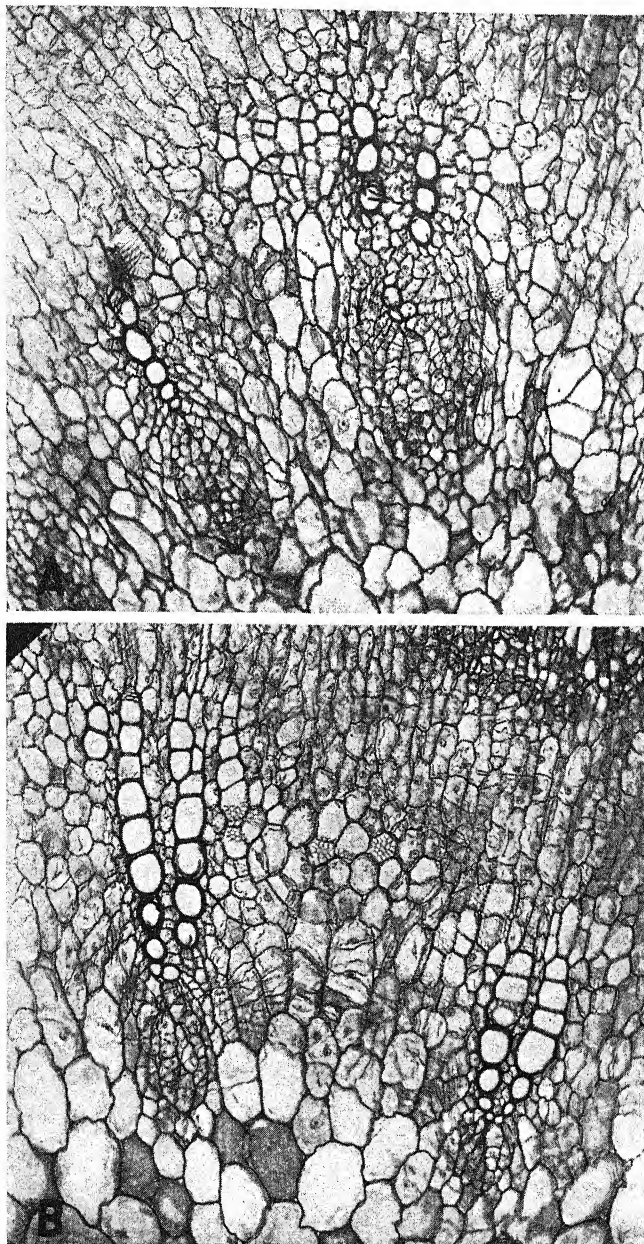


FIG. 19.—Eighty hours after treatment. *A*: section 1.3 mm. from treated surface. *B*: 1.6 mm. distant from surface. In both, the pith, ray, and xylem parenchyma cells are active and many of the cells have matured as large pitted tracheids across the rays and adjacent to the xylem.

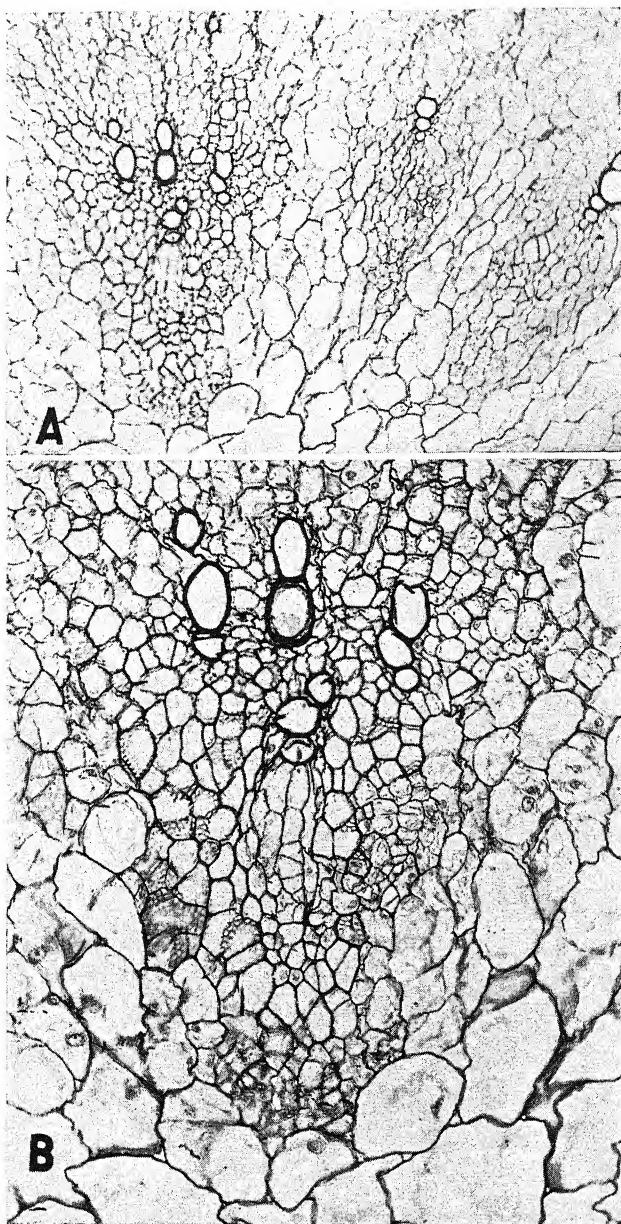


FIG. 20.—One hundred ten hours after treatment, section 1.4 mm. below treated surface. *B*: enlarged view of region at left in *A*. Active ray, xylem parenchyma, and pith cells. Some derivatives have matured as pitted tracheids; others as phloem elements; many still highly meristematic.

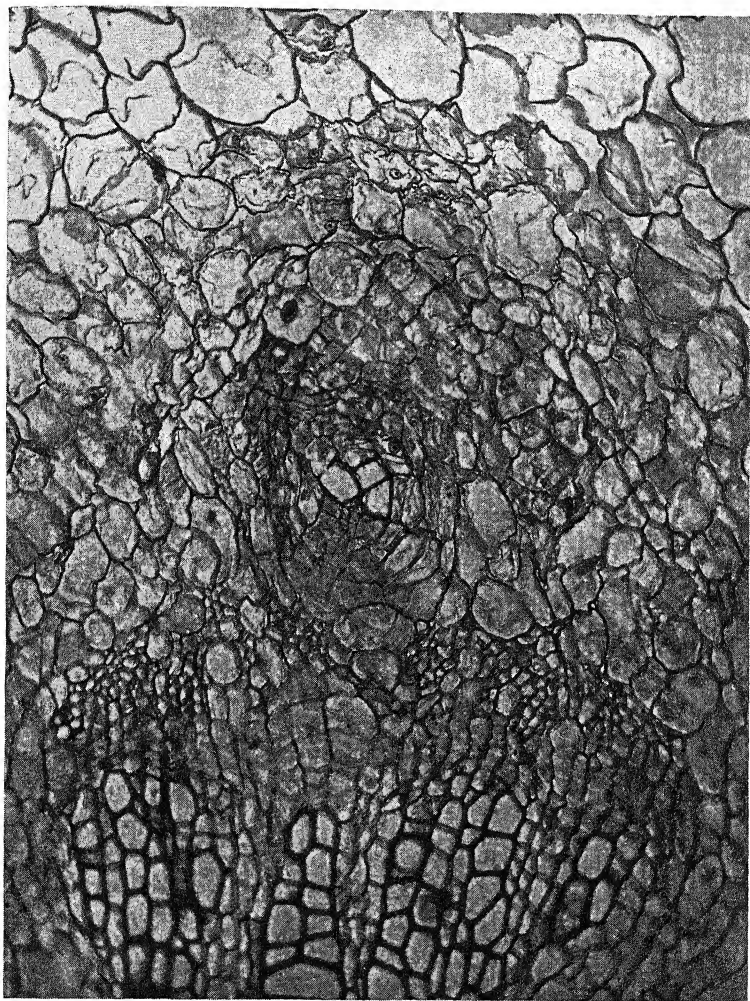


FIG. 21.—One hundred ten hours after treatment, section 5 mm. below treated surface. Tissues other than endodermis and primary phloem show little response to treatment. In the primary phloem a vascular strand has differentiated. At center of strand are large pitted tracheids surrounded by meristematic cells some of which would later differentiate as tracheids, and others continue to divide. At upper left of this bundle sieve tubes are differentiated just within the cells of the pericycle which approach maturity (*cf.* fig. 16).

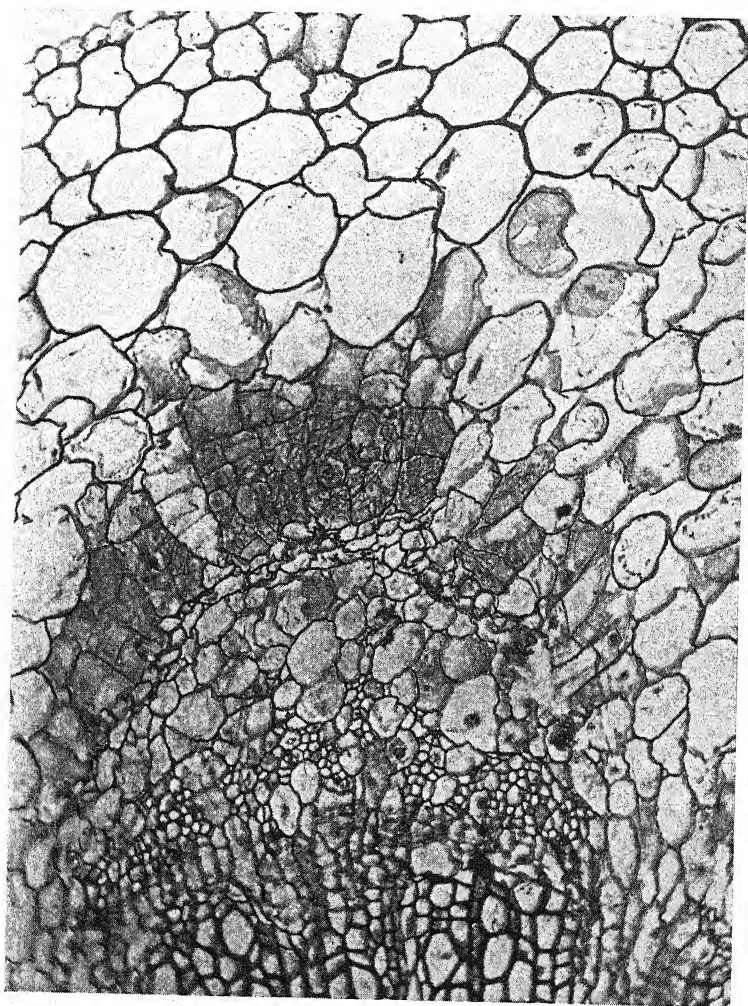


FIG. 22.—Same section as fig. 21 but of another vascular bundle. In this instance a vascular strand is being initiated in the proliferated endodermal cells. Walls of original endodermal cells clearly evident at left. One cell has divided tangentially only, another in several planes (*cf.* fig. 23).



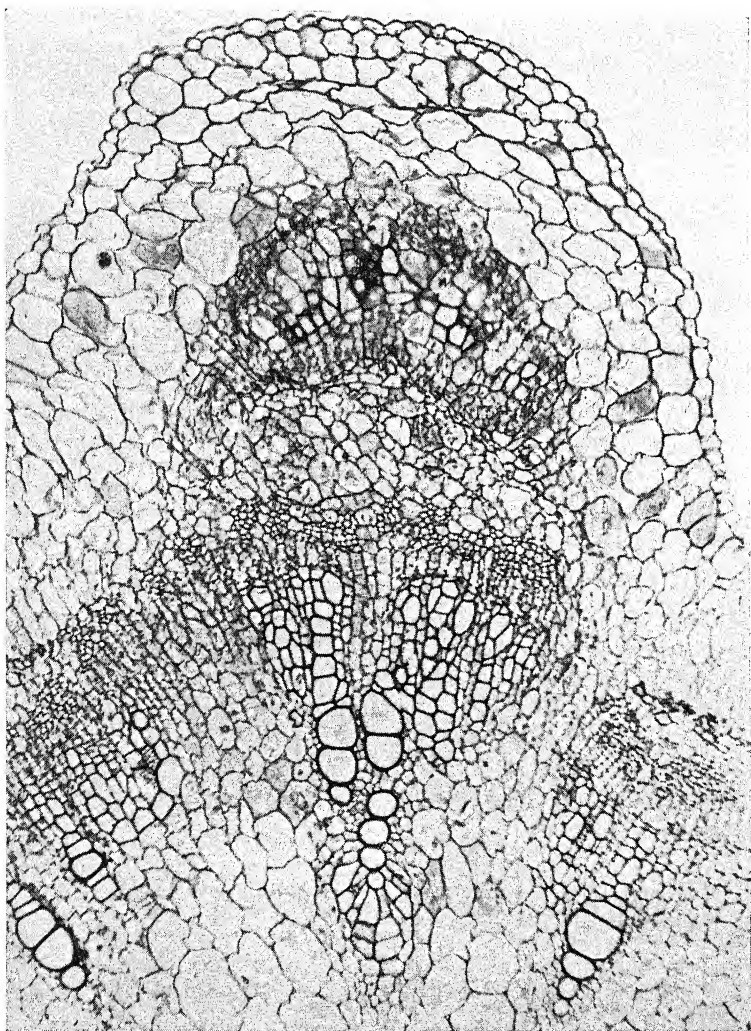


FIG. 23.—Same stem as figs. 21 and 22, section at slightly higher level (3 mm. below treated surface). Vascular elements derived from cells of proliferated endodermis. The whole group is in a more advanced stage than as shown in fig. 22. This section and that shown in figs. 21 and 22 were taken below region in which the apical crown of adventitious roots developed. It is especially noteworthy that in these sections the ray cells show only slight meristematic activity although the pith cells adjacent to protoxylem and metaxylem have proliferated and many derivatives matured as tracheids.

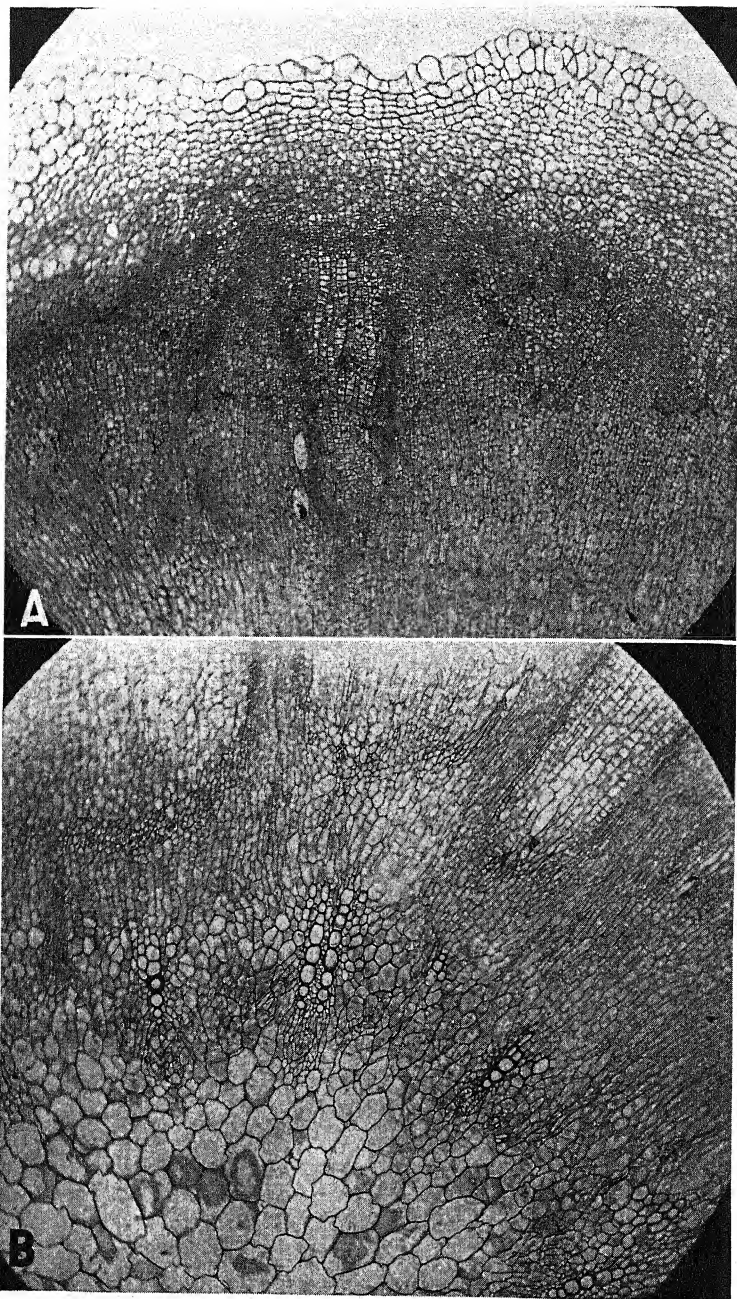


FIG. 24.—*A*: 114 hours after treatment, section 1.2 mm. below treated surface about through middle of zone in which adventitious roots developed, showing two young roots with proliferated endodermal and cortical tissues exterior to them and phloem between and at the sides. *B*: 116 hours after treatment, at approximately same level in stem as *A*. Relation of young roots to phloem, rays, and xylem is obvious.

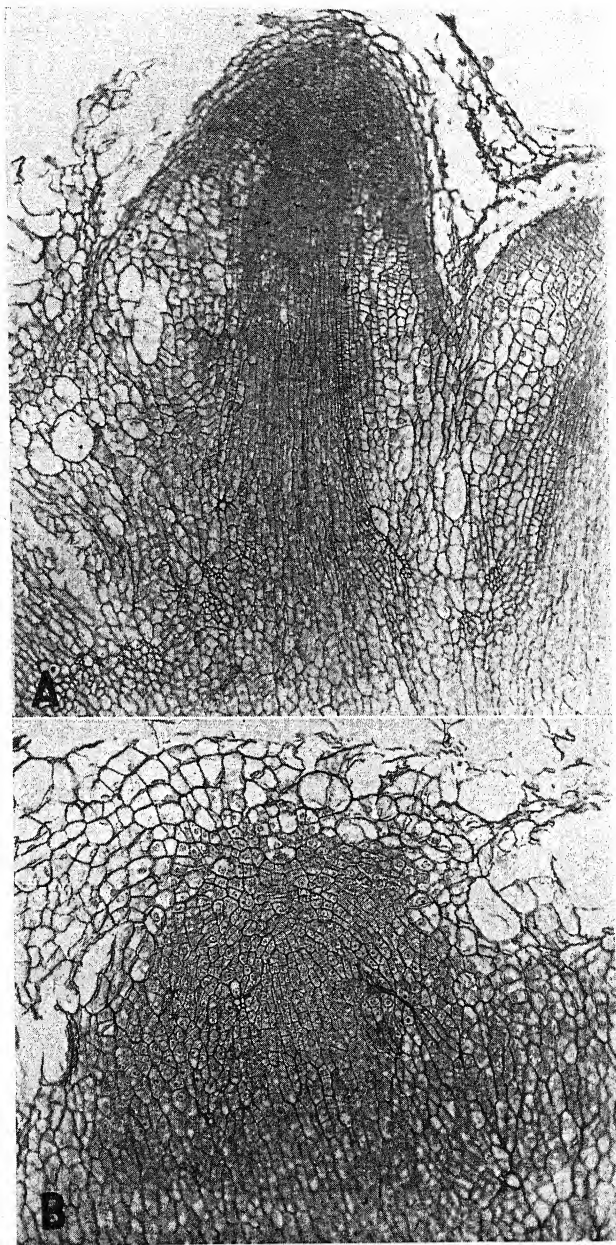


FIG. 25.—*A*: 120 hours after treatment, median longitudinal section of adventitious root. Central core is derived from the ray, its outer portions from the phloem, as indicated by the groups of sieve tubes stretching throughout its length, its tip capped with endodermal and cortical tissue. *B*: 110 hours after treatment, early stage in development of apical histogen. The scattered, denser, irregular patches at either side of central core are sieve tubes of the proliferated phloem.



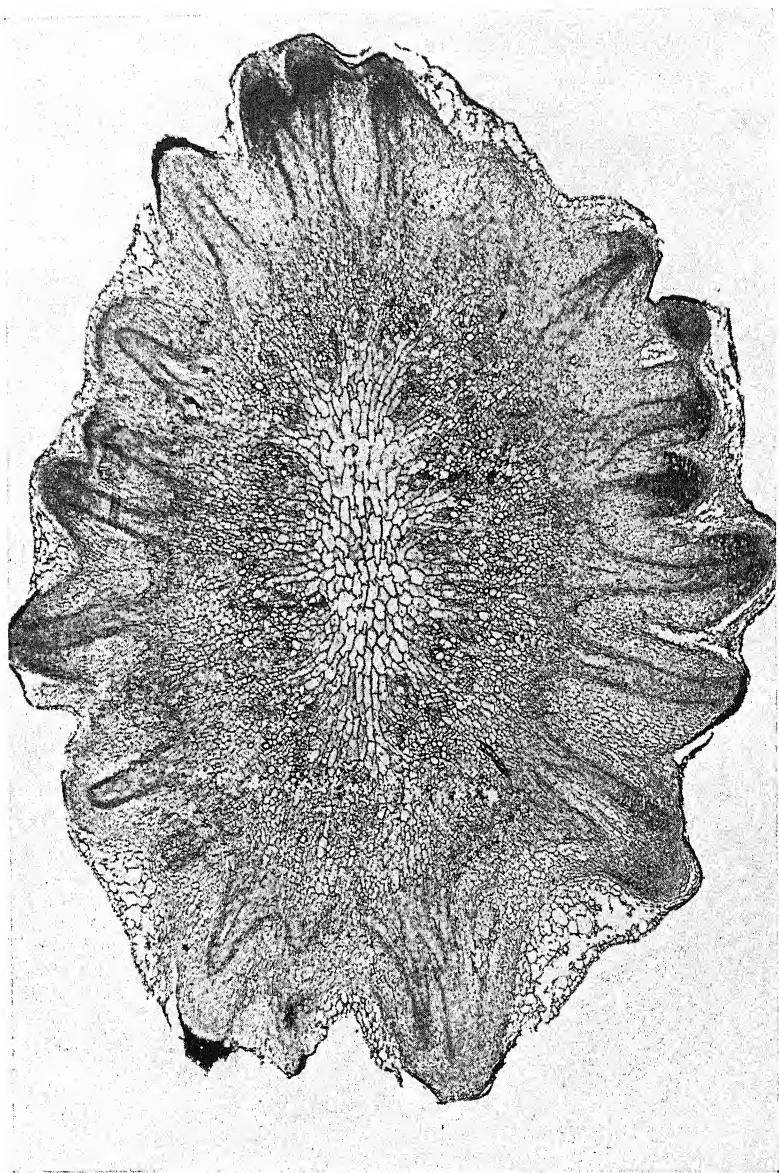


FIG. 26.—One hundred sixty-eight hours after treatment, section about 1.5 mm. below treated surface and through crown of apical roots. The relation of roots to rays and proliferated phloem is shown. Most of the cortical tissues are dead and broken; proliferated endodermal cells are still living and intact at many places over the phloem and over some of the root tips. Pith has proliferated and from the derivatives have come large tracheids, vascular strands, and meristematic zones, bands, and islands (see fig. 28 A).

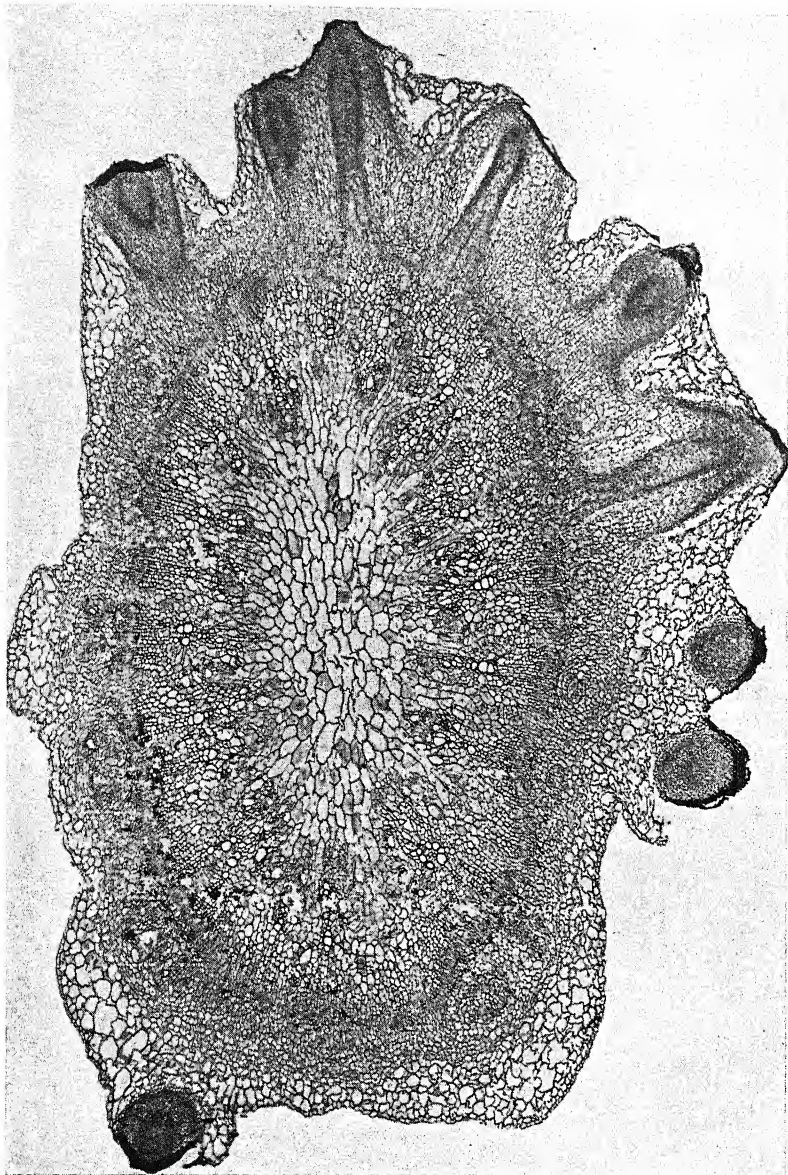


FIG. 27.—Same stem as fig. 26, section at lower level, just below main crown of roots but through several roots at the top. Phloem does not show the extensive proliferation and radial extension that it does in the root zone, nor does the endodermis. Vascular strands have differentiated from the primary phloem in many of the bundles. Rays have proliferated, as has also the pith, and from the derivatives have come vascular strands, tracheids, and masses of meristematic cells.

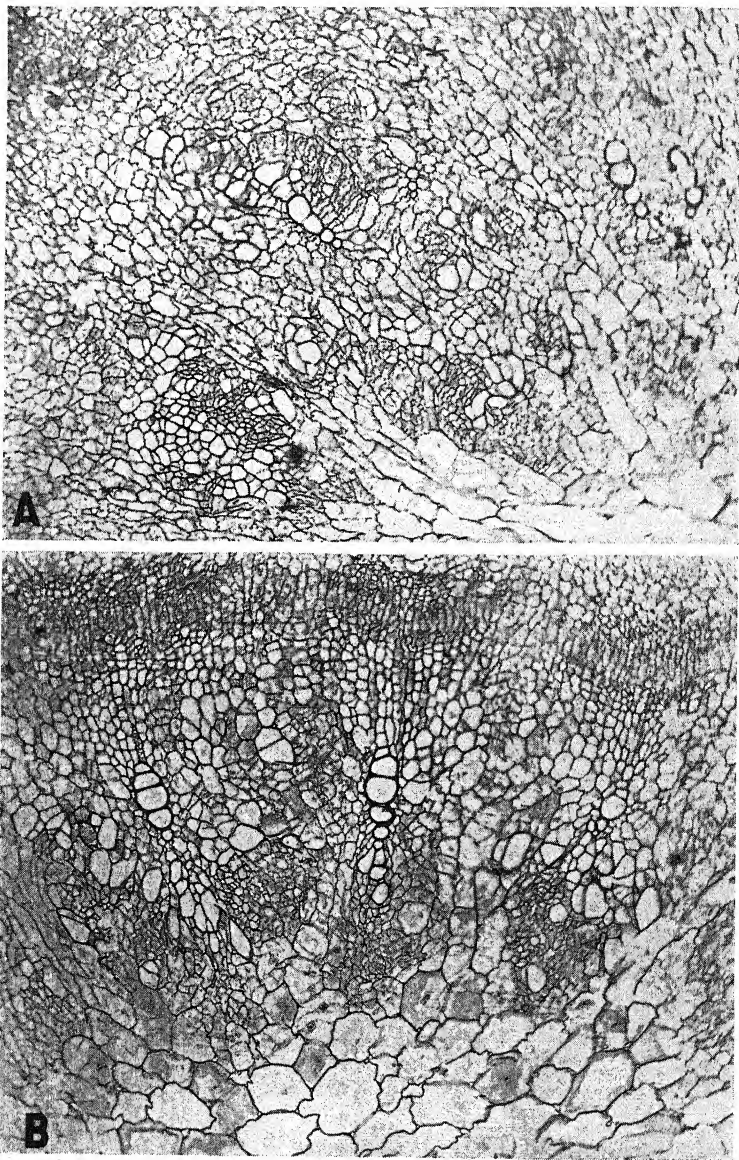


FIG. 28.—*A*: detailed view of portion of rays and pith, as in fig. 26. Following proliferations of the cells of both tissues, a confused mass of vascular elements, parenchymatous cells, and highly meristematic areas has developed. *B*: detail of same regions but at same level as fig. 27. Three points of primary xylem are shown. Outward from them are secondary xylem, cambium, and phloem; inward are large tracheids, vascular strands, meristematic masses, and pith cells showing first divisions and others as yet undivided.

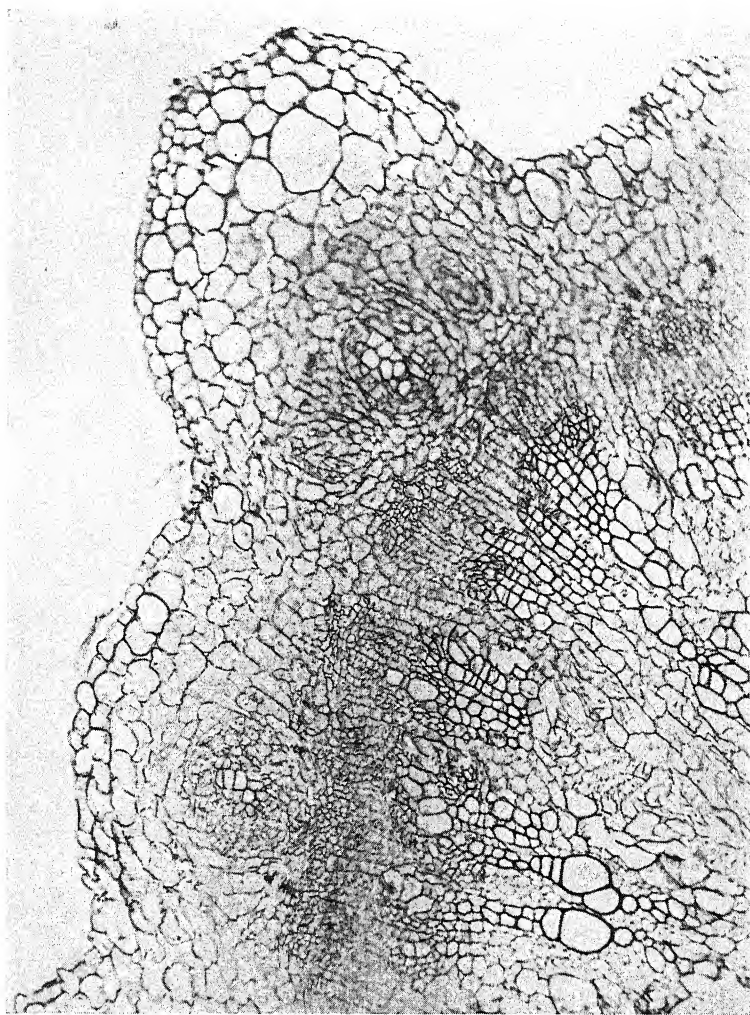


FIG. 29.—Same stem as figs. 26–28, section 6 mm. from treated surface. Vascular strands present in region of primary phloem. These extend for more than 3 cm. down this particular stem. Rays and pith about as in fig. 28.

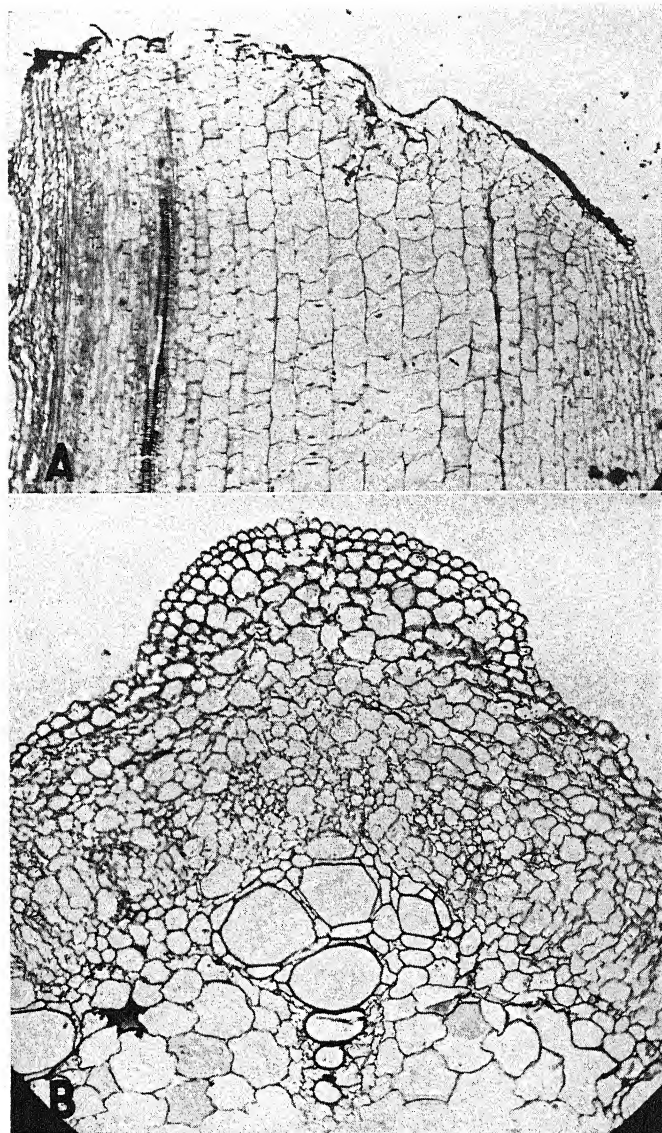


FIG. 30.—*A*: 12 days after treatment of cut surface with pure lanolin only, median longitudinal section of stem. A phellogen has developed across entire stem adjacent to cut surface. *B*: 15 days after treating cut surface with pure lanolin, section 1 mm. below treated surface. None of tissues shows unusual degree of proliferation.



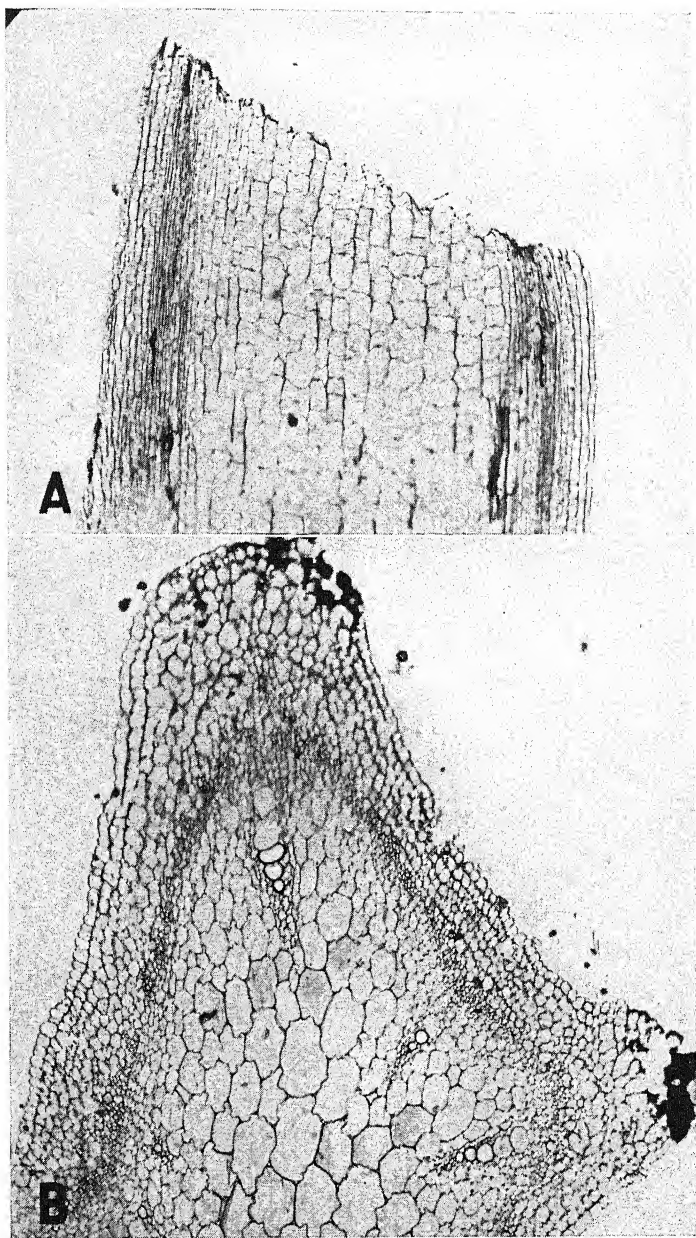


FIG. 31.—Five days after decapitation, the cut surface untreated. *A*: median longitudinal section. *B*: section 1 mm. below cut surface. Cells of some of the tissues are dead, but most of them are alive and show varying degrees of maturation. About half of the stems decapitated and untreated formed no callus and dried out much as those here illustrated; the others formed a callus, as shown in figs. 32 and 33.

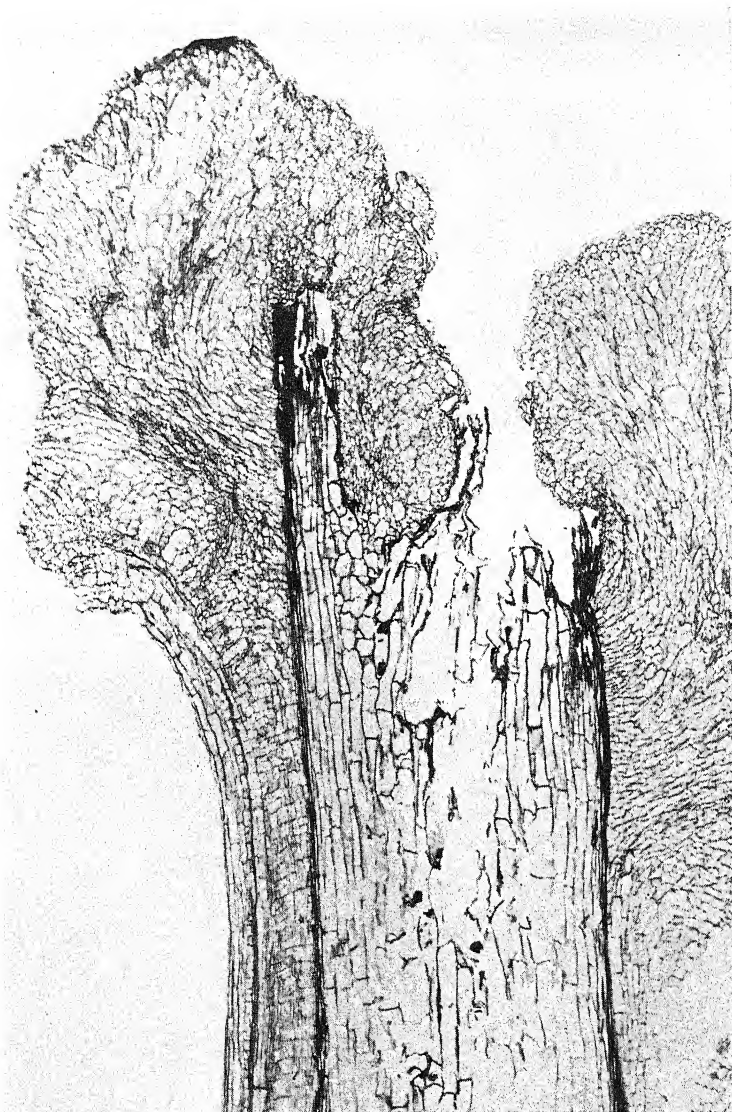


FIG. 32.—Fifteen days after decapitation, the cut surface untreated. Many pith cells are dead and dried out. The callus developed almost entirely from proliferated phloem. At left, below the callus, endodermis is visible as single line of cells three or four cell layers within the epidermis and just outside the outermost black line, the pericycle. Within this is a broad zone of phloem which abuts a narrow band of xylem. In some specimens the endodermal cells at the base of and below the callus show several divisions in the tangential plane.

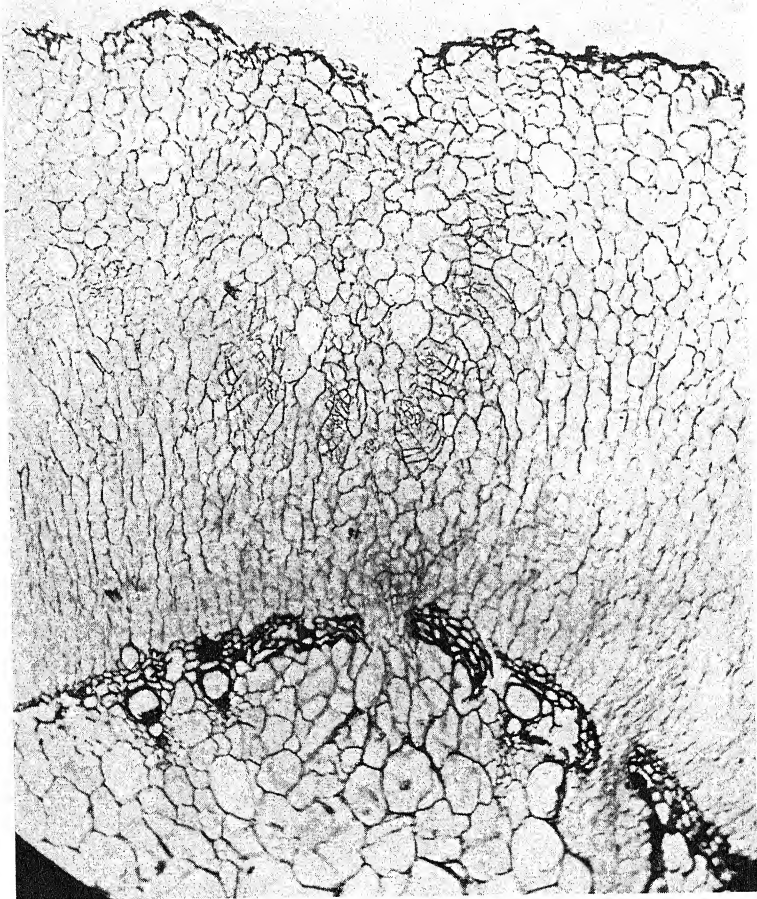


FIG. 33.—Fifteen days after decapitation, the cut surface untreated, section about 1 mm. below original cut surface through callus tissue. Most of the xylem present at time of cutting is dead. A few of the rays, and in places the pith, have proliferated. Most of the callus is from the phloem. Derived cells have matured into tracheids, phloem elements, and large parenchymatous cells. Meristematic activity is general throughout the mass of parenchymatous cells. Outermost cells have suberized.



of the phloem of the original bundles of the stem. A cambium, continuous with the cambium in the adjacent vascular bundles, is differentiated. Apparently it arises from the outermost layer of the inner core of cells derived from the ray rather than the surrounding cells derived from the phloem. New tissues are added from an apical histogen, and as growth proceeds the young root pushes out through the covering layers of endodermal and other cortical cells. It matures in much the same way as do secondary roots.

As has been stated previously, decapitated but otherwise untreated stems generally form a terminal callus over the cut surface, whereas those treated with the lanolin mixture form large irregular tumors. Such calluses are derived mainly through a pronounced proliferation and subsequent partial or complete differentiation of the cells of the phloem. In some instances some of the cells of the ray and of the outer regions of the pith also proliferate. Two specimens examined showed activity on the part of the endodermis, but this is very slight in comparison with the activity manifested by endodermal cells when stimulated with indoleacetic acid. After a time some of the outermost cells of the callus suberize, others mature as large pitted tracheids or phloem elements, some remain as large parenchymatous cells, and many continue actively meristematic. Old calluses show irregularly organized vascular strands scattered through them.

### Discussion

In many respects the overgrowths produced on Kidney bean resulting from the application of indoleacetic acid in lanolin resemble the tumors produced by bean and other plants treated with a growth substance extracted from *Bacterium tumefaciens*, as has been pointed out by BROWN and GARDNER (3). SMITH (12), and SMITH, BROWN, and McCULLOCH (11) have drawn a parallel between the reaction of plant tissues to infections by *B. tumefaciens* and cancerous growth in animals. Many others have also developed a similar viewpoint. In following out this concept, SMITH and his co-workers not only studied the structure and development of tumors resulting from injection of *B. tumefaciens*, but also conducted a number of experiments in which a series of chemical compounds was applied to vari-

ous plant tissues. The cellular and histological responses of the treated plants were studied. While it is not now our purpose to state specifically that the responses of plant tissues to indoleacetic acid are duplicates of those resulting from inoculations with *B. tumefaciens*, yet the similarities are many, as BROWN and GARDNER have indicated. The works of SMITH and his collaborators furnish the most extensive account of the histological details available to us.

The following excerpts from SMITH, BROWN, and McCULLOCH will give, in part, an indication of their point of view.

"The tumor originates in meristem, usually in the cambium region. It may perish within a few months or continue to grow (parts of it) for years.

"The tumor consists, or may consist, not only of parenchyma cells but also of vessels and fibers, i.e., it is provided with a stroma which develops gradually as the tumor grows. A proliferating tumor usually contains not only meristem but pitted vessels and sieve tubes; it may also contain wood fibers, but does not always.

"The tumor sends out roots (tumor strands) into the normal tissues. These may extend for some distance from the tumor—how far is not known. These strands consist of meristem capable of originating medullary rays, tracheids, and sieve tubes. In the daisy the strand passes through the protoxylem region of the stem. It is rich in chloroplasts. . . . A considerable part of it consists of unripe, actively vegetating cells.

"In the daisy the infiltrations are not through the vessels, but between them in a tissue offering little resistance to intrusion, i.e., the region occupied by the thin-walled, delicate spiral vessels.

"In the substance of these deep-lying strands secondary tumors develop. These gradually rupture their way to the surface.

"There are no true metastases in crown gall, but this does not, to our mind, militate against the comparison, for whether a cancer shall be propagated by floating islands of tissues, or only by tumor-strands, appears to be a secondary matter depending on the character of the host tissues rather than on the nature of the disease. The essential element is the internal stimulus to cell division.

"The stimulus to tumor development comes from the presence of the parasite within certain of the cells. Apparently it is not in all."

In brief, SMITH (12) assumed that *B. tumefaciens* lived mainly as an intracellular parasite, but the immediate or proximate cause of the phenomena of cell proliferation "must be the chemical or physical action of enzymes or other substances produced or activated by the bacteria as a result of their metabolism, with a corresponding reaction on the part of the plant." Some of the cells of the host might show definite reactions to the foreign organism although they were not in contact with the bacterial cells and such response might be situated at some considerable distance from them.

RIKER (10), BANFIELD (2), and others have suggested that the bacterial organism concerned in the development of crown gall is intercellular. According to BANFIELD, who worked with cane gall, the "cells with which the bacteria are in contact undergo a more or less rapid cytolysis. In early stages of gall formation those cells at a distance from the bacteria divide rapidly. Subsequently intercellular penetration may occur, and in turn those cells at a distance may be incited to extensive division. Eventually cell division ceases, extensive intercellular penetration by the bacteria continues, and the gall degenerates."

Each hypothesis, whether it postulates the foreign organism as intracellular or intercellular, includes the suggestion that the living cells of the host react to some substance generated by the bacteria, and that the bacteria need not be in direct contact with the cells which have been stimulated to divide.

The histological responses of the cells of the bean plant to indoleacetic acid show many developmental patterns which are almost exact duplicates of those SMITH, BROWN, and McCULLOCH have shown for crown gall. Those which follow the application of the indoleacetic acid are somewhat more pronounced. It is not entirely clear whether SMITH considered the tumor strands of crown gall as strands of cells which interpenetrated other tissues and then enlarged and differentiated subsequently or whether strands of cells *in situ* were stimulated, became meristematic and their derivatives differentiated into the many and varied forms he illustrated. Certain it is that his photomicrographs clearly indicate the latter to have been the case. We have obtained such changes by the use of indoleacetic acid, the resultant structures having the same appearance as

tumor strands. The prolonged meristematic activity of some of the tissues and the continued differentiation of aberrant structures from some of the regions, such as the pith, following treatment with indoleacetic acid, warrant much further experimentation and study. Whether such behavior on the part of the tissues is to be likened to tumor growths in animals must await subsequent findings.

What, in cytological and physiological detail, may be the character of the response of cells to stimulation by growth substances awaits further investigation. JONES (7, 8, 9) has recently suggested that there is a "possibility that missing genes due to corresponding deficiencies in certain parts of both members of a chromosome pair result in a chromosomal unbalance and this brings about unregulated growth. . . . The well-known effect of x-rays, radium, hormones, and other powerful physical and chemical agents in bringing about unregulated growths seems conclusively to be due in some way to their action upon the chromosomes resulting in the loss of parts or of whole chromosomes." SMITH, BROWN, and McCULLOCH showed that in the development of crown gall there are many and varied amitotic and mitotic divisions of the nuclei. In our present work we have not yet carried out cytological investigations. Such investigations would be of great value, although it does not seem necessary to assume that there must be chromosomal changes or any type of mutation to account for the histological developments observed. As has been pointed out, the several main tissue systems differ widely in their degree and type of response to stimulation. As far as could be determined from the studies made, none of the types of cells developed was fundamentally different from cells occurring in bean plants grown under the usual conditions of culture and environment, although the sizes, numbers, and relative proportions of the various types of cells show wide departures from the conditions found in such plants. One of the most striking responses following the application of the lanolin mixture is the tendency of cells to become highly meristematic and for some of the derived tissues to remain in such condition for long periods of time. Further details in this connection will be given when tumors older than 168 hours are discussed.

Among other responses, the pronounced development of vascular

strands from the tissues of the endodermis, primary phloem, pith, and other regions, which under the general cultural conditions of the bean are not generally encountered, emphasizes again the degree to which differentiated cells retain their totipotential capacities.

In discussing crowngall, SMITH summarizes some of his concepts on growth as follows: "Growth is the normal function of cells. They are always multiplying when they are not inhibited by one thing or another. Growth, then, if this view is correct, comes about not by the direct application of stimuli, but indirectly *by the removal of various inhibitions*. Under normal conditions the physiological brakes are on at all times, more or less, in both plants and animals, and only when they are entirely or largely removed in particular areas do we observe an unlimited cell proliferation resulting in the hasty and peculiar growths known as neoplasms or cancers. . . . The inhibition remover we are in search of is *one that acts locally, disturbing tissue equilibriums within limited areas*." SMITH has considerably more to say concerning the relative importance of chemical and physical factors as stimuli or as removers of inhibition. Any critical evaluation of the various views advanced concerning the phenomenon of growth, the rather vague term unregulated growth, and the like, are beyond the scope of the present paper.

Perhaps it will be possible eventually to approach somewhat more closely an explanation of some growth responses of cells through the use of an increasing list of growth substances available as chemically pure compounds. But as yet, even though it may be possible to account for the behavior of cells, it is not possible to account physiologically for the developmental patterns of tissues, largely because so few detailed patterns related to specific stimuli are available for critical examination.

### Summary

1. The living cells of the stem of Red Kidney bean are responsive to applications of indoleacetic acid in lanolin. The mixture used consisted of 30 mg. of indoleacetic acid per gram of pure lanolin.
2. Young bean seedlings were decapitated by cutting the stem off squarely at the top of the second internode of the primary axis. The

cut surface was left untreated or smeared once with a thin coating of pure lanolin or the lanolin-indoleacetic acid mixture.

3. Gross observations extending over several weeks were made on the untreated and treated stems without removing them from the plant. Histological studies were made of material collected and preserved at 6-hour intervals up to a total of 168 hours. The several tissues responded variously to treatment, depending in part upon their distance from the surface of application of the mixture.

4. The epidermal cells undergo a few divisions in the radial plane and enlarge somewhat after treatment.

5. The cells of the cortical parenchyma enlarge somewhat and those near the endodermis become meristematic. Starch grains and chloroplasts disappear from them when activity is initiated. After a variable number of hours the outermost cells die and in the vicinity of the root tips they are pushed aside and disrupted. At distances more than 5 mm. from the surface of application the cortical cells show little histological change but may lose their content of chlorophyll and starch for a time; at later stages these again appear.

6. The cells of the endodermis are highly responsive to indoleacetic acid. Nuclear division is greatly speeded up shortly after application. Walls subsequently form in all planes, especially the tangential. Within 168 hours cells 5 cm. below the point of application are highly meristematic. The derived cells differentiate as xylem and phloem elements and large, often multinucleate, parenchymatous cells. Many remain meristematic and still others give rise to adventitious roots. Especially over the vascular bundles, long proliferating strands of vascular tissue are developed. These frequently enlarge to the extent that they rupture the tissues exterior to them and appear as tumors in rows along the stem.

7. The cells of the pericycle proliferate slightly and mature as parenchymatous cells, or, if some distance from the lanolin mixture, as thick walled blunt fibers.

8. The parenchyma of primary phloem shows the same general type of response and subsequent differentiation of tissues as does the endodermis. In addition some of the cells derived from it form a part of the outermost tissues of the apical crown of adventitious roots.

9. The parenchyma of the secondary phloem undergoes marked proliferation. Most of the tissues composing the outer portion of the apical crown of adventitious roots are derived from it. Other cells mature into parenchymatous tissue, tracheids with simple pits, sieve tubes, and companion cells.

10. When stems are decapitated and otherwise untreated, calluses may be formed. These are derived mainly from the proliferation of the parenchymatous cells of the primary and secondary phloem parenchyma.

11. Decapitated stems treated with lanolin only, thereafter generally enlarge but slightly. The tissues mature and a weak phellogen is formed just beneath the cut surface. Numerous chloroplasts develop in the cortical cells which also contain large deposits of starch.

12. The cambium divides actively; its derivatives may continue their meristematic activity over a considerable period of time, later maturing as various phloem and xylem elements or continuing as meristematic islands or zones from which such elements continue to be derived.

13. Near the surface of application the cells of the ray adjacent to the xylem proliferate greatly. Many of their derivatives mature as tracheids, and commonly a confused system of vascular strands intermingled with highly meristematic areas is developed from them. This confused mass of tissues may persist and continue development for many weeks. The cells adjacent to the phloem and just within the pericycle also proliferate greatly, elongate radially, and in conjunction with the phloem cells flanking them form the principal portions of the apical crown of adventitious roots. The histogens of such roots seem to originate mainly in cells derived from the rays. This is true also for the cambium and xylem of the adventitious roots.

14. The pith cells proliferate greatly. Activity is first noticeable directly adjacent to the surface of application. As time goes on activity progresses down the stem next to the elements of the protoxylem and metaxylem. At still later stages activity may extend to the center of the stem. Maturation of the derived cells results in large pitted tracheids adjacent to the elements of the primary xylem and the formation of a confused mass of vascular strands, scattered

vascular elements, large parenchymatous cells, and meristematic zones and islands where the pith formerly was. These tissues continue to develop for a long period of time and account for most of the overgrowths which come to overtop the apical tumor and eventually attain a large size.

15. The histological developments following the application of indoleacetic acid closely resemble many of those associated with crown gall produced by *Bacterium tumefaciens*.

16. The most striking feature shown by stems treated with lanolin mixture is the very great speeding up of nuclear divisions, especially in cells near the surface of application. Such divisions result in a multinucleate condition of many of the cells. Later cell division occurs and the derived cells may remain highly meristematic for long periods of time.

17. It does not seem essential to assume that the developmental patterns expressed by tissues following treatment necessarily result from an effect altering the genetic composition of the chromosomes to the degree that parts of chromosomes or whole chromosomes have been lost.

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## IS PROTOPLASM ELASTIC?<sup>1</sup>

HENRY T. NORTEN

(WITH ONE FIGURE)

A controversy exists as to whether protoplasm behaves like a true fluid (1) or whether it behaves like an anomalous fluid (2). If protoplasm is a true fluid it should possess one viscosity value; that is, the value should be independent of the pressure or shearing stress. On the other hand, if protoplasm is an anomalous fluid it will possess an infinite number of viscosity values, the value obtained depending upon the pressure or shearing stress.

According to SEIFRIZ (2), fluids which possess structural aggregates are elastic. Hence if it can be demonstrated with certainty that protoplasm is elastic, it can be inferred that protoplasm is built up of intermeshed, linear, crystalline units. The following data demonstrate that the "viscosity" of cytoplasm of *Zygnema* sp. varies with the shearing force and is therefore elastic.

The method used was based on Stoke's law which governs the rate of movement of a particle through a fluid. According to this law:

$$L = 2cg(D - d)a^2t/9\eta \quad (1)$$

in which  $L$  is the distance moved in the time  $t$ ,  $g$  the gravity constant,  $(D - d)$  the difference in specific gravity between the moving particle and the surrounding medium,  $a$  the radius of the particle,  $\eta$  the viscosity of the medium, and  $c$  a constant which may be determined from the following equation:

$$c = 4.024rn^2 \quad (2)$$

<sup>1</sup> Contributions from the Department of Botany and the Rocky Mountain Herbarium of the University of Wyoming, no. 159.

in which  $r$  is the radius of the centrifuge in meters and  $n$  the number of revolutions per second. From equation (1):  $L \propto ct$ , and  $\eta \propto 1/L$ . Hence  $\eta \propto 1/ct$ . From equation (2):  $c \propto n^2$ . Hence  $\eta \propto 1/tn^2$ . From a consideration of the last proportion: if protoplasm is a true fluid, the same value of  $\eta$  should be obtained as long as the product of  $tn^2$  remains constant. If protoplasm is elastic,  $\eta$  should

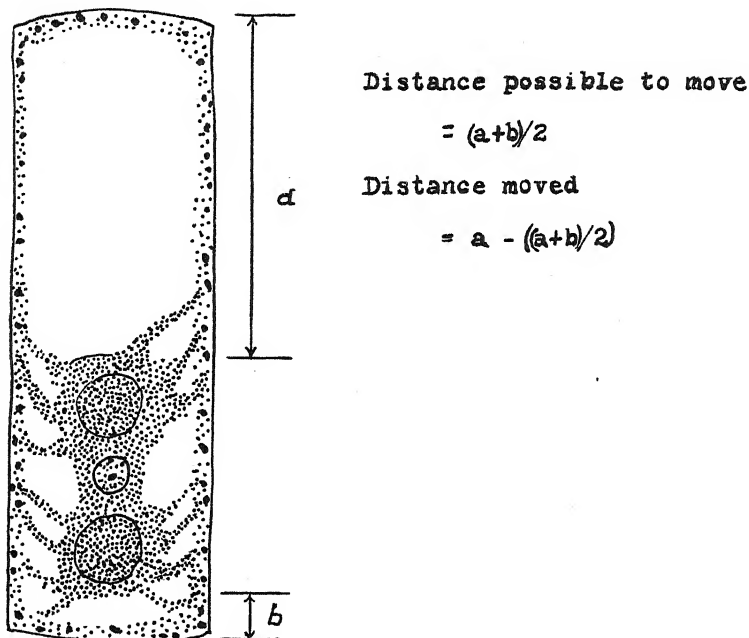


FIG. 1

be lower when  $n$  is small and  $t$  large even though the product of  $tn^2$  remains constant.

In the following experiments, filaments of *Zygnema* sp. were centrifuged at different speeds with a hand driven centrifuge, but for any one experiment the product of  $tn^2$  was constant. Following centrifugation the filaments were mounted in a 1 per cent solution of eosin which likewise contained 1 cc. of acetic acid per 100 cc. of solution. It was observed that mounting in this solution maintained the chloroplasts for hours in the same position as they were a moment after centrifugation.

The distances the chloroplasts move in response to centrifugal force were determined as shown in figure 1, and the results are given in table I.

In experiment number one, the filaments were centrifuged for too long a period to bring out any significant difference between the cells centrifuged with a force of  $471.9 \times$  gravity and those centrifuged with a force of  $118.0 \times$  gravity.

TABLE I  
MOVEMENT OF CHLOROPLASTS IN RESPONSE TO DIFFERENT  
SHEARING FORCES

EXPERIMENT NO.	NO. OF CELLS MEASURED	C FORCE $\times$ GRAVITY	SECONDS CENTRIFUGED	TC	DISTANCE POSSIBLE*	DISTANCE MOVED* (FLUIDITY)	RELATIVE VISCOSITY (1/FLUIDITY)
1	54	471.9	90	42471	0.89	0.86	1.16
	63	118.0	360	42471	0.88	0.869	1.15
	72	29.5	1440	42471	0.811	0.722	1.38
	57	13.6	3120	42471	0.781	0.612	1.63
2	84	118.0	240	28320	0.895	0.602	1.66
	97	29.6	960	28320	0.793	0.287	3.48
	119	7.4	3840	28320	0.807	0.273	3.66
3	51	118.0	240	28320	0.80	0.49	2.04
	54	29.6	960	28320	0.711	0.209	4.78
4	54	118.0	240	28320	1.023	0.946	1.06
	93	29.6	960	28320	0.895	0.535	1.87
	111	7.4	3840	28320	0.944	0.362	2.76
5	83	679.9	30	20397	0.896	0.724	1.38
	101	170.0	120	20397	0.882	0.608	1.64

\* In relative units.

It might be expected that the corresponding viscosity values in experiments 2, 3, and 4 would agree more closely. For example, in experiment 2 a value of 1.66 is recorded for the cells centrifuged with a force of  $118.0 \times$  gravity whilst in experiment 4 a value of 1.06 is recorded for those centrifuged with a similar force. There are several reasons for these discrepancies. First, the cells vary in length, and in general the longer the cell, the more readily will the chloroplast move. The column "Distance possible" gives an indication of the average length of the cells which were measured. Second, the physio-

logical state of the cell at the time of experimentation must be considered. The rate with which the chloroplast moves through the cytoplasm will vary from morning to afternoon, from afternoon to evening, and from day to day. The "viscosity" of the cytoplasm will vary with time, but perhaps more important is the change in the relative specific gravity of the chloroplast, which will depend upon the amount of starch stored within and immediately around the chloroplast. To eliminate as far as possible the variations due to time, the centrifuging in each experiment was conducted within a period which did not last over two hours.

From a consideration of the relative "viscosity" values obtained in this experiment, the following conclusions are justified: First, no single viscosity value can be ascribed to the cytoplasm of *Zygnema* cells. There are an infinite number of viscosity values and the value obtained will depend upon the shearing force—the greater the force used, the lower the apparent viscosity. Second, the cytoplasm of *Zygnema* cells is elastic.

### Summary

1. Filaments of *Zygnema* sp. were centrifuged with different forces, but in each experiment the product of time centrifuged and the square of the number of revolutions per second remained constant.
2. The experiments demonstrate that no single viscosity value can be ascribed to the cytoplasm of *Zygnema* cells. The value obtained depends upon the shearing stress—the lower the stress, the higher the apparent viscosity.
3. It can be concluded that the cytoplasm of *Zygnema* cells is elastic.

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## OFFSPRING OF A SELF-POLLINATED REVERSED CARPELLATE PLANT OF *MORUS ALBA*<sup>1</sup>

JOHN H. SCHAFFNER

In 1929, the writer published the results of a breeding experiment with self-pollinated seeds obtained from a staminate plant of *Morus alba* L. which had several branches showing sex reversal to femaleness (2). The progeny from these seeds consisted of pure staminate individuals, pure carpellate individuals, and individuals with mixed sex expression at the first blooming period. It was evident, therefore, that in the white mulberry an individual originally determined as a pure male had the potentialities to produce offspring of both sexes without the introduction of any new hereditary units from the outside. Sex reversal had been seen previously in carpellate plants but no trees were available at this time for experiment, although young trees were being grown in order to make a definite study of reversal to maleness in carpellate individuals. These young trees were transplanted from the greenhouse to the botanic garden, and in 1932 a carpellate plant showed several small branches with reversal to maleness, developing a few staminate catkins. These branches were inclosed with some carpellate branches with cloth to keep out all foreign pollen, and a small quantity of selfed seeds was obtained. These seeds were dried for a few days and then planted in the greenhouse. The seedlings developed vigorously in a long-light photoperiod during the winter, and the next spring (1933) they were planted in rich soil in the garden. In the meantime, the spring of 1934 was very dry and the original parent tree, now ten years old, bloomed abundantly and showed a decided increase in the number of reversed branches and the number of staminate catkins and flowers. Nearly all of the main branches of the tree now developed some staminate catkins. There was a decided gain in maleness in this tree since the spring of 1932. In the regions of sex reversal many of the catkins were poorly developed, with part of the flowers either vestigial or distorted. In some cases ovularies were found with imperfect anthers developing in their sides.

<sup>1</sup> Papers from the Department of Botany, The Ohio State University, no. 379.

Of the plants obtained from the selfed seeds twenty-four survived, and in the spring of 1935 two trees had attained reproductive maturity and each produced a few pure carpellate catkins. None of the other trees developed any flowers and several of them were still quite small, because of several years of unfavorable conditions.

In the spring of 1936, most of the trees were from 6 to 12 feet high, and by May 8 twenty of them were in bloom, while the smallest trees still developed no flowers. Of the twenty trees that bloomed, five were pure staminate in sex expression, eight were pure carpellate, four were decidedly staminate but produced some carpellate flowers, one was decidedly carpellate but had several catkins with some staminate flowers, and two were of decidedly mixed sexual expression, mostly with catkins showing both carpellate and staminate flowers. The eighteen trees which were either pure carpellate or pure staminate in sex expression or very decidedly female or male thus showed an equal number for each sex, nine female and nine male. Although there were only five pure staminate trees to eight pure carpellate, the difference is not significant because of the small numbers; but the general results are significant, since they show that in *Morus alba* an hereditary constitution, which was originally determined as female, can without any addition from outside sources produce offspring which at their first blooming period are pure male and pure female, just as an hereditary constitution which was originally determined as male can produce both pure male and pure female offspring without any additional heredity from outside sources. The two trees which were pure female at their first blooming period in 1935 continued as pure females in 1936 and produced an abundant crop of fruit.

It is evident that no hypothesis based on the notion of a homozygous-heterozygous sex-determining mechanism of Mendelian sex genes or factors will explain these results. Both the individuals which are originally determined as pure male and pure female contain the potentialities for both sexes, and the determination of the one sex or the other is then necessarily dependent on a differential physiological state brought about through some physico-chemical condition present at the time in the cells concerned, whether it be the original determination in the egg or a later determination or sex reversal in the vegetative buds or tissues. In dioecious plants the orig-

inal sex determination probably mostly takes place by a swing of the physiological sex balance, in the egg, either before fertilization, at the time of fertilization or the formation of the zygote, or immediately after the fertilization process has been completed. In some special cases, however, as for example in *Arisaema triphyllum* (1), it appears that the sex balance is originally always in the direction of maleness, the young plants all being staminate, while in the mature condition the individuals are pure staminate and pure carpellate. It is established through experimental means that the mature individuals of *A. triphyllum* are balanced equally in respect to sex determination and sex constancy, the reversal from one sex to the other being induced as easily in one direction as the other.

It is evident that it is impossible to determine the sex reactions of a species by observations on only a single reproductive period, whether the first or any subsequent one. To determine the true sexual nature of the individual or race, all available extreme ecological environments must be tried before a formula can be established, either for the sexual constitution or for the ordinary Mendelian gene constitution concerned in the determination of secondary sexual characters. *Morus alba* evidently contains a complete complement for both sexes in every individual. The stability or instability of differentiated systems is not a condition peculiar to sexual phenomena but is in evidence in the tissues and organs in both the higher plants and animals. In some cases remarkably stable systems are differentiated from the heredity complex while in others the differentiated tissues may be dedifferentiated and a new differentiation process initiated without adding to or subtracting from the complex of hereditary units. These reversals and dedifferentiations, which play such a prominent part in sexual phenomena, indicate that the sex determinations are caused by physiological or physico-chemical states in the protoplasm and not by hereditary differentials through the shifting of chromosomes or other protoplasmic organs; and from this point of view, the results of the experiments with *Morus alba* become intelligible.

It is evident that if the physiological view of sex determination is correct, different species, having distinctive, general, hereditary constitutions, may give quite different results from reversed selfed plants. The following six types or categories of simple sex expression are the-



oretically possible: (1) both males and females may produce both sexes, as is the case in *Morus alba*; (2) males may produce only males and females only females as YAMPOLSKY found in *Mercurialis annua*; (3) males may produce both sexes while females produce only females; (4) females may produce both sexes while males produce only males; (5) both reversed males and females (as well as ordinary cross pollinations) may produce offspring all of which are originally determined as males, as appears to be the case in *Arisaema triphyllum*; (6) both reversed males and females may produce offspring all of which are originally determined as females.

### Summary

1. A reversed carpellate tree of *Morus alba* was self-pollinated and from the seed produced twenty-four young trees were grown. At the first blooming period only two trees bloomed and these were pure carpellate. At the second blooming period, a year later, twenty of the twenty-four trees bloomed and of these five were pure staminate; eight (including the two carpellate trees of the previous season) were pure carpellate; four were decidedly staminate but each one had some carpellate flowers; one was decidedly carpellate but had several catkins with some staminate flowers; and two were of decidedly mixed sex expression.

2. These results correspond with those obtained several years earlier from a reversed staminate tree from whose self-pollinated seed a progeny was developed also consisting of pure males, pure females, and individuals of mixed sex expression. The results from the two experiments show that the dioeciousness or unisexuality of *Morus* is not caused by hereditary differentials, like an "xy" or "wz" allosome set or by any of the newer factor hypotheses, but by a physico-chemical or physiological condition, since both male and female individuals are potentially bisexual. This conclusion is confirmed by the frequent sex reversals which take place in both young and old trees.

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## CURRENT LITERATURE

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*Growth Hormones in Plants.* Authorized English translation of *Die Wuchsstofftheorie und ihre Bedeutung für die Analyse des Wachstums und der Wachstumsbewegungen der Pflanzen.* By P. BOYSEN JENSEN. Translated and revised by GEORGE S. AVERY, JR. and PAUL R. BURKHOLDER, with the collaboration of HARRIET B. CREIGHTON and BEATRICE A. SCHEER. McGraw-Hill Book Co., New York. 1936.

The German text by P. BOYSEN JENSEN, which appeared in 1935, attempted to present the chief lines of investigation and the main results of research done in the last 25 years. The views of various investigators were presented objectively, and experimentally founded facts and hypothetical views were distinguished. Particular attention was given to the fundamentals of the growth substance theory.

The new book is more than a translation and revision, as those terms usually indicate. It is essentially a new text which takes advantage of all the information in the German text and adds results of investigations to date from all over the world. There are 40 new figures and illustrations, and approximately 200 new citations. No attempt has been made to present a literal translation of the German edition. The reader will frequently wonder whether the versions belong to JENSEN or to the American authors. To the student this will make little difference; to the scientist, it may.

The book has been reorganized, certain chapters rearranged, and parts condensed or expanded. The final chapter of the German volume is broken up and distributed through earlier chapters. New features are a summary at the end of each chapter, a substantial index, and historical reviews presented with a series of diagrams.

The historical review will help students to understand how subject matter is developed through years of effort. Sixty-four illustrations enrich the volume and make it more understandable by those less familiar with this new subject. The book is literally filled with references, going back to the time of SACHS and DARWIN. The real starting place, however, is the published work of BOYSEN JENSEN in 1910, with conclusions based on experimentally founded facts. Using the coleoptile of *Avena*, decapitated and otherwise, BOYSEN JENSEN showed that the conduction of the stimulus causing phototropic curvature takes place on the shaded side by the downward movement of a growth-promoting substance. Since that time, great advances in our knowledge have been made, but the book makes clear that there are still many points needing further investigation. Scientists throughout America will certainly welcome this English text.—P. W. ZIMMERMAN.

*Die Süßwasser-flora Mitteleuropas, Heft 15: Pteridophyten und Phanerogamen, unter gleichzeitiger Berücksichtigung der wichtigsten Wasser- und Sumpfwächse des ganzen Kontinents von Europa.* By H. GLÜCK. Jena: Gustav Fischer, 1936. Pp. xx+486. Figs. 258.

During the past three decades the researches of H. GLÜCK upon the life histories and ecological relationships of the European Alismaceae and various species of *Utricularia*, also his foliar and floral studies of various other aquatics and near-aquatics, have received an important place in botanical literature. The present work is the fifteenth and numerically the final volume in the series of brilliant treatises on the fresh-water flora of central Europe being issued and in part written by Dr. ADOLPH PASCHER of Prague. (Certain volumes dealing with the simpler plants are still in process of completion.)

Unlike the fungi and several other groups of lower plants which have numerous species that are more or less cosmopolitan, the plants of the present text mostly display pronounced restrictions of range. Thus the European plants included by GLÜCK (his treatment is not limited to central European plants) are largely absent from North America. His descriptions and illustrations will prove interesting to American botanists, however, because of the numerous comparable or analogous habitat forms to be found in our own flora. The text abounds, as do some of GLÜCK's earlier writings, in *formae*, varieties, and hybrids. A *forma* with GLÜCK is often what American taxonomists would designate merely as a "state." To a local (central European) ecologist, the inclusion of every discernible habitat state as a *forma* or even *subforma* may seem desirable, if only as a teaching aid. For taxonomists, however, the dignifying of innumerable local "states" with names implying formal taxonomic status may well inspire misgivings.

The volume is small and will fit many pockets. The figures are simply drawn but exceedingly clear and helpful. Unfortunately, the paper used is of an impermanent type such as is all too common in scientific books of the present day. It is much to be desired that works of major scientific value, such as GLÜCK's really is, may be issued, even if in a small supplementary edition, in a permanent form for libraries.—E. E. SHERFF.

*Monographie du Genre Cestrum L.*, part I. By PIERRE FRANCEY. *Candollea* 6:46-398. 1935.

In a work which won the DECANDOLLE prize in 1934, FRANCEY offers the first part of a monographic arrangement of the Solanaceous genus, *Cestrum* L.

The treatment opens with a preface and this is followed by two parts, general and systematic. The former presents a historical view of *Cestrum*, distinguishes it from the allied genus *Sessea*, discusses its morphology, and gives in detail an account of the geographical distribution, which is almost exclusively American. The systematic section, which is of course the main part, contains a total of 205 species, the first 28 being placed in Section *Habrothamnus*

(Endl.) Schlecht. and the rest in Section Eucestrum Dunal. The descriptions, which are especially full, are in Latin throughout, as are also the keys, while the supplementary text is in French. Types are not specially indicated, and in the case of some species this is apt to prove vexing. In general, however, high standards of excellence are uniformly maintained. Among monographs which have appeared during the present decade, FRANCEY'S will easily rank as one of the very best.—E. E. SHERFF.

*Experimental Enzyme Chemistry.* By HENRY TAUBER, Burgess Publishing Co., Minneapolis, 1936. Pp. 118. \$3.50.

The rapid advances made in recent years in the experimental study of enzyme chemistry are summarized briefly in a mimeoprint book by Dr. HENRY TAUBER, of the New York Homeopathic Medical College and Flower Hospital, New York. No attempt is made to present the more theoretical aspects of the subject; the author has tried chiefly to summarize the recent progress in methods and results of experimental studies in the field of enzyme reactions. The introductory chapter includes brief reference to many subjects, such as temperature effects, pH, specificity, mass action, development of equations, inhibition, inactivation, kinetics, antiseptics, mechanism of enzyme reactions, enzyme syntheses, and the preparation of enzyme materials.

The succeeding chapters are devoted to groups of enzymes: esterases, proteolytic enzymes and peptidases, amidases, carbohydrases, catalase, oxidizing enzymes, the flavin oxidation system, carbonic anhydrase, the zymase complex, and luciferase. The material is usually presented in brief paragraphs that state what has been done, with citation of the papers containing the original results. Extensive bibliographies accompany each chapter.

Although it contains but 118 pages, this volume is condensed and contains considerable information. For those who wish a summary of recent experimental techniques and results, it will be a helpful guide. It does not take the place of the original literature, but it does cite much of it, so that one may quickly become familiar with the most important recent advances in any part of the field.—C. A. SHULL.

*Statistical Methods for Research Workers.* By R. A. FISHER. 6th ed. Oliver & Boyd, London, 1936. Pp. xiii+339.

*Statistical Methods in Biology, Medicine and Psychology.* By C. B. DAVENPORT and MERLE P. EKAS. John Wiley & Sons, New York, 1936. Pp. xii+216.

Those who desire to study their problems by statistical methods have a choice of two excellent manuals offered to them in new editions. The first is the sixth edition of FISHER'S statistical methods for research workers; the second is the fourth edition of the work by DAVENPORT and EKAS. Both of these works have been long and favorably known, and description is superfluous. Only a few changes are noted in FISHER'S new edition, which was also true of all

earlier editions. Section 22, ex. 15.1, presents a complex test on homogeneity in data with hierarchical subdivisions; section 26 makes reference to WORKING and HOTELLING'S formula for sampling error of values estimated by regression; and section 29.2 directs attention to an extended use of successive summation in fitting polynomials. Table *z* has also been extended to the 0.1 per cent level of significance. Earlier editions need not be discarded for these few additions.

The work by DAVENPORT and EKAS has been thoroughly revised, FISHER'S methods for small samples being incorporated into it, but it still retains the general character of former editions. Either manual provides excellent guidance into the statistical analysis of observations made on large or small numbers of individuals or groups.—C. A. SHULL.

# THE BOTANICAL GAZETTE

March 1937

## PHYSIOLOGICAL STUDIES WITH THE NITROGEN- FIXING ALGA, NOSTOC MUSCORUM

FRANKLIN E. ALLISON, SAM R. HOOVER, AND HERMAN J. MORRIS

(WITH NINE FIGURES)

### Introduction

The ability of certain species of blue-green algae to utilize free nitrogen when grown in pure culture was not conclusively proved until the year 1928, although much early work with mixed cultures of algae and bacteria had suggested this possibility. DREWES (15) grew *Anabaena variabilis*, *Nostoc punctiforme*, and a second species of *Anabaena* in pure culture on a nitrogen-free medium and obtained nitrogen fixation. ALLISON and MORRIS (3, 4) also isolated a blue-green alga from the soil and demonstrated its nitrogen-fixing ability. At the time it was thought to be a species of *Anabaena*, but later work has shown that it is a *Nostoc*. Further studies with this organism were reported in brief recently (1), and the present paper gives additional data with regard to the conditions which favor nitrogen fixation by this common soil organism. COPELAND (14) reported that certain, but not all, thermal strains of the Myxophyceae, including strains of *Oscillatoria princeps*, *O. formosa*, *Spirulina labyrinthiformis*, and *Phormidium laminosum*, isolated from nitrogen-deficient warm springs, utilize atmospheric nitrogen.

Some of the endophytic blue-green algae which live in symbiosis with higher green plants (*Blasia*, *Cavicularia*, *Anthoceros*, *Azolla*, *Cycas*, *Gunnera*, and *Encephalartos*) have also been shown to be able

to secure their nitrogen from the air. VOUK and WELLISCH (34) discuss the available information on this subject. MOLISCH (25) obtained bacteria-free cultures of *Nostoc* from *Blasia* and *Cavicularia* and demonstrated that they grew as well on nitrogen-free media as in the presence of potassium nitrate, but did not report nitrogen analyses. VOUK and WELLISCH likewise obtained good growths of *Nostoc* from *Anthoceros* and *Blasia*, and of *Anabaena azollae* on nitrogen-free media. WINTER (35) also has recently shown that *Nostoc punctiforme*, isolated from five host plants and grown in pure culture (two out of the five cultures), fixed up to 1.95 mg. nitrogen per 50 cc. of culture solution. Fixation did not occur in the absence of a suitable sugar source.

### Observations

#### DESCRIPTION OF ORGANISM

Extensive microscopic studies, carried out in part in cooperation with Dr. FRANCIS DROUET of the University of Missouri, have shown that the characteristics of the organism used in these investigations agree in every essential respect with those of *Nostoc muscorum* Ag. ex Born et Flah. The identification of the organism proved rather difficult because it grows somewhat differently in artificial culture solutions than in nature. In soil where the moisture is limited, the colonies may contain few trichomes (filaments), whereas in solution cultures the organism sometimes continues to grow for long periods, largely as trichomes, often lying nearly parallel, as in the case of *Anabaena*.

The spores are usually somewhat smaller than most of the descriptions of the organism specify, but it is very probable that the smaller spore size is the more common, judging by the observations of BRISTOL (7), LOWE and MOYSE (23), and ourselves. Wide variation in spore size is at least a well recognized characteristic (6, 19, 33). The colonies, as we observed them on soil kept in Erlenmeyer flasks plugged with cotton, were less gelatinous and more inclined to spread in thin surface films than other workers have reported. This was undoubtedly due to the more abundant supply of moisture in the soil and air under our conditions. Attempts to secure strains of

*Nostoc muscorum* from other workers for direct comparisons of growth characteristics were unsuccessful.

A description of the organism as we found it, grown chiefly in liquid and agar cultures using inorganic, sugar-free media, is as follows. A few cultures were also grown on soil.

PLANT MASS.—On soil the colonies are gelatinous-membranaceous, nearly round at first, commonly 1–5 cm. in diameter but expanding irregularly, especially in the presence of an abundance of moisture, and are dark olive green to yellowish brown. On agar the colonies usually spread more rapidly to form a thin surface layer. In liquid media thin, widely spreading light or dark green films form, which if undisturbed remain largely at the surface, being supported in part by the bubbles of gas given off during photosynthesis.

TRICHOMES.—In young cultures the trichomes or filaments are frequently motile, nearly straight, and may lie more or less parallel; usually flexuous and densely entangled later. Sheaths are plainly visible surrounding the older forms. In old cultures these filaments often break up, leaving the individual cells essentially free but held together in jelly-like masses. Motile trichomes or hormogones may form as the result of the repeated division of these individual cells, by the breaking of the older trichomes at the heterocysts or elsewhere, or by spore germination.

CELLS.—Vegetative cells are of varying shapes, spherical, barrel-shaped, cylindrical, or quadrate, 2.5–4  $\mu$  broad, 3.5–5  $\mu$  long. Young cells are usually barrel-shaped but older cells are commonly round or oval.

HETEROCYSTS.—Formed abundantly, almost round and when mature 5–7  $\mu$  in diameter. Both the intercalary and terminal types are common, usually one to three per trichome but occasionally as many as twenty being observed. The intercalary type is almost round and when mature 5–7  $\mu$  in diameter. The true terminal heterocysts are formed terminally, are somewhat pointed, and have one polar nodule instead of two as in the intercalary type.

SPORES.—Often formed abundantly in older cultures, especially where these are allowed to dry out slowly. Develop in catenate series between heterocysts. In some cases nearly all vegetative cells may be converted into spores. Usually oval or nearly spherical but



may be somewhat angular. Size 4-6  $\mu$  wide and 4-10  $\mu$  long. The more common size is 4-5  $\mu$  wide and 6-7  $\mu$  long. Walls smooth. Color yellow or yellowish brown when mature.

HABITAT.—Isolated from the surface of moist shaded soil and later from a small pool of water in the District of Columbia. Preserved specimens of this organism will be placed in the U.S. National Herbarium, the New York Botanical Garden, and the Farlow Herbarium at Harvard University.

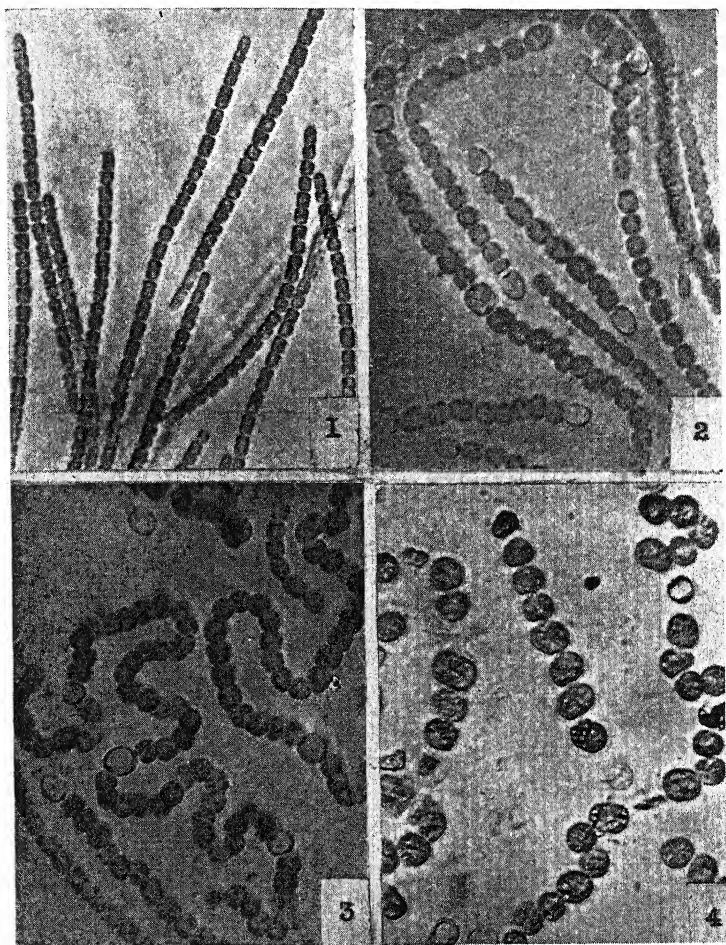
Photomicrographs of the organism are shown in figures 1-8.

#### METHODS

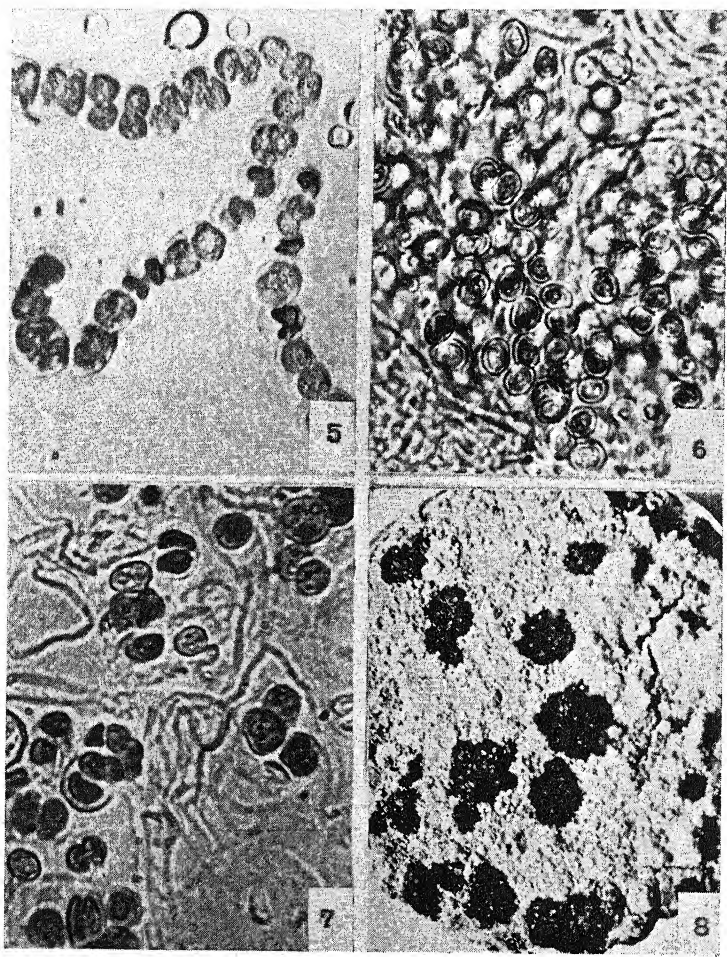
The experiments were carried out over a period of about six years, using a variety of media and growth conditions. The primary purpose of the work was, in fact, to determine the optimum conditions for growth and nitrogen fixation and some of the factors which affect the rate of fixation. Only representative experiments of a large number carried out are reported here. The media used in each experiment, as well as the specific growth conditions, are given in connection with each table, since uniform experimental conditions were not used throughout the work. Artificial light, supplied by a bank of 60 to 100 watt Mazda lamps, was used as the light source. The daily period of illumination varied from 12 to 24 hours.

Erlenmeyer flasks of 250 cc. capacity, fitted with cotton plugs and containing 100 cc. of media, were used in growing the unaerated cultures; 500 cc. pyrex gas washing bottles, ordinarily containing 100 cc. of media, were used for the aerated cultures. Aeration was accomplished by passing compressed air, containing the desired percentage of  $\text{CO}_2$ , through concentrated  $\text{H}_2\text{SO}_4$  to remove any ammonia or oxides of nitrogen and then through a solution of  $\text{HgCl}_2$ . The culture vessels were usually maintained at a temperature of 24°-27° C. by keeping them immersed in a water bath to a depth of about 1 inch.

All media were sterilized in the autoclave for a period of 15 to 20 minutes at 15 pounds' pressure, except where glucose was present, in which case the time was 10 minutes at 10 pounds' pressure. All the usual precautions against contaminations were exercised. Inoculations were made from a young culture, grown on a sugar medium in



FIGS. 1-4.—*Nostoc muscorum* Ag., photomicrographs of living unstained cells from liquid inorganic nitrogen-free media: Fig. 1, young actively growing, motile trichomes, 20 days old. Fig. 2, trichomes that have come to rest and formed terminal and intercalary heterocysts; motile trichomes (without heterocysts) also present; age of culture 27 days. Fig. 3, stage in growth of organism just prior to breaking of trichomes at heterocysts to form motile hormogones; continued cell division of non-motile trichomes, such as shown in fig. 2, produces these contorted trichomes; culture 27 days old. Fig. 4, old trichomes with individual cells essentially free but held together in jelly-like masses, a few starting to germinate; culture 10 months old.  $\times 780$ .



FIGS. 5-8.—*Nostoc muscorum*: Fig. 5, cell division in old vegetative cells seen occasionally; each cell may develop into a trichome; inorganic, nitrogen-free medium; culture 10 months old.  $\times 780$ . Fig. 6, spores thoroughly desiccated, shown imbedded in mass of fungus filaments.  $\times 780$ . Fig. 7, germinating spores (as in fig. 6) 10 days after addition to fresh medium.  $\times 780$ . Fig. 8, colonies on soil. Approximately normal size.

order the more readily to detect contaminants. Usually a very small quantity of young trichomes from a culture, grown in a liquid medium, was transferred by means of a sterile wire. This method is not entirely satisfactory because it is impossible to add the same number of cells to the various flasks. Inoculations may also be made by means of a pipette if a culture having an abundance of young motile organisms is used. An attempt was made to use this system, but it was not very satisfactory because motile trichomes commonly become non-motile and form films and later gelatinous masses very quickly.

All nitrogen analyses were made by the macro-Kjeldahl method, using  $\text{H}_2\text{SO}_4$ ,  $\text{CuSO}_4$ , and  $\text{K}_2\text{SO}_4$  in the digestion flasks. The algal cells and medium in which they had grown were analyzed together unless otherwise stated.

#### MEDIA

*Nostoc muscorum* was found to grow readily upon a wide variety of media such as are commonly used in growing *Azotobacter*, *Rhizobium*, and green algae; yet it is not always easy to grow it in the laboratory for long periods so as to obtain large masses of the organism, unless its growth requirements are understood. Very slow growth or death may result from a variety of causes, but numerous experiments have shown that the development of an unfavorable pH is usually the primary factor. The shift may be toward either the acid or alkaline side, depending upon the medium used and the growth conditions. These will be discussed later.

Tables 1-8 give in part the results of the studies on media, but for the sake of brevity they will not be discussed individually in this connection. All the results, together with numerous observations and preliminary experiments not reported here, are summarized later.

**AERATED CULTURES.**—The following medium was found to be very suitable for the growth of cultures aerated with air containing 1 per cent  $\text{CO}_2$ :  $\text{K}_2\text{HPO}_4$ , 0.5 gm.;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.2 gm.;  $\text{NaCl}$ , 0.2 gm.;  $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ , 0.1 gm.;  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , 0.005 gm.;  $\text{CaCO}_3$ , 1-5 gm.;  $\text{H}_2\text{O}$ , 1000 cc. Natural humic acid or synthetic iron humate (2, 11, 12) at the rate of 5 p.p.m. may sometimes be substituted with ad-

vantage for the ferric chloride. The NaCl is probably not essential. This medium usually has a pH of 8 or higher after autoclaving, but when aerated with 1 per cent CO<sub>2</sub> in air the pH drops to near 7.

TABLE 1  
EFFECT OF NATURAL HUMIC ACID ON NITROGEN FIXATION

TREATMENT	NITROGEN ADDED PER 100 CC. (MG.)	FIRST EXPERIMENT* (UNAEERATED)		SECOND EXPERIMENT (AERATED 1% CO <sub>2</sub> IN AIR)		
		TOTAL NITROGEN (MG.)	AVERAGE NITROGEN FIXED (MG.)	TOTAL NITROGEN (MG.)	AVERAGE NITROGEN FIXED (MG.)	FINAL pH
Check (basal medium only).	0	8.45 9.68	9.07	8.02 8.70	8.36	7.2 7.6
Asparagin . . . . .	5	14.27 13.42	8.85	9.92 9.67	4.80	7.2 7.3
KNO <sub>3</sub> . . . . .	5	.....	.....	9.40 9.43	4.42	7.7 7.7
Humic acid, 10 p.p.m. ....	0.04	14.23 15.69	14.92	.....	.....	.....
Humic acid, 20 p.p.m. ....	0.1	.....	.....	17.40 12.07	14.64	7.0 7.1
Humic acid, 100 p.p.m. ....	0.42	18.98 16.41	17.28	.....	.....	.....
Humic acid, 20 p.p.m. + as- paragin . . . . .	5.1	.....	.....	19.15 20.91	14.93	7.1 6.9
Humic acid, 20 p.p.m. + KNO <sub>3</sub> . . . . .	5.1	.....	.....	12.28 12.50	7.29	7.3 7.2
Humic acid, 100 p.p.m. + asparagin . . . . .	5.42	20.23 25.10	17.25	.....	.....	.....

\* Cultures nearly dead at end of experiment.

Basal medium: First experiment, K<sub>2</sub>HPO<sub>4</sub>, 0.5 gm.; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.2 gm.; NaCl, 0.2 gm.; CaSO<sub>4</sub>·2H<sub>2</sub>O, 0.1 gm.; FeCl<sub>3</sub>·6H<sub>2</sub>O, 0.005 gm.; CaCO<sub>3</sub>, 1.0 gm.; glucose, 10 gm.; H<sub>2</sub>O, 1000 cc. pH 7.8. Second experiment, sucrose substituted for glucose.

Light: First experiment, artificial, 18-hour day, 200 foot candles; second, 12-hour day, 300 foot candles.

Incubation period: First experiment, 85 days (11/27/29-2/20/30); second, 78 days (3/21/30-7/7/30).

When media similar to the preceding but without CaCO<sub>3</sub> were used, the growth was almost invariably considerably slower, owing in part at least to the lower pH resulting.

UNAERATED CULTURES.—The medium is usually not satisfactory for the growth of the alga in unaerated cultures, but the omission of

TABLE 2  
VARIOUS MEDIA FOR USE IN AERATED AND UNAERATED CULTURES

TREATMENT (ADDITIONS PER 100 CC. OF MEDIUM)	UNAERATED				AERATED (1% CO <sub>2</sub> IN AIR)			
	NITRO- GEN FIXED (MG.)	AVER- AGE (MG.)	FINAL PH	FINAL CELL CONDI- TION	NITRO- GEN FIXED (MG.)	AVER- AGE (MG.)	FINAL PH	FINAL CELL CONDI- TION
Check (basal medium I only).....	1.55		7.3	Normal	5.06		6.6	Normal
	1.43	1.49	7.3	"	5.62	5.34	6.6	"
CaCO <sub>3</sub> , 20 mg.....	2.29		8.0	Poor	7.06		6.7	"
	1.72	2.01	7.8	"	6.97	7.02	6.7	"
CaCO <sub>3</sub> , 50 mg.....	2.32		8.0	"	8.50		6.9	"
	1.87	2.10	8.0	"	5.70	7.10	7.0	"
CaCO <sub>3</sub> , 100 mg.....	2.10		8.0	"	10.18		7.4	"
	1.98	2.04	8.0	"	9.43	9.81	7.6	"
CaCO <sub>3</sub> , 100 mg. + glu- cose, 1%.....	2.14		8.3	Fair	7.58		7.5	"
	1.24	1.69	8.2	"	7.10	7.34	7.3	"
CaCO <sub>3</sub> , 100 mg. + su- crose, 1%.....	2.14		8.1	Poor	17.34		7.2	"
	2.14	2.14	8.0	"	19.92	18.63	7.0	"
Glucose, 1% (no CaCO <sub>3</sub> )	0.06		6.8	Normal	1.32		6.4	Fair
	0.25	0.16	6.8	"	0.73	1.03	6.4	"
Sucrose, 1% (no CaCO <sub>3</sub> )	2.38		7.2	"	12.40		5.9	Normal
	2.22	2.30	7.3	"	11.19	11.80	6.0	"
Basal medium II only..	2.65		6.8	"	2.08		6.6	"
	1.45	2.05	6.8	"	2.38	2.23	6.4	"
Basal medium II + CaCO <sub>3</sub> , 100 mg.....	2.97		8.0	Poor	10.36		7.0	"
	2.55	2.76	8.3	"	10.51	10.44	7.2	"

Basal medium I: K<sub>2</sub>HPO<sub>4</sub>, 1.0 gm.; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.2 gm.; NaCl, 0.2 gm.; CaSO<sub>4</sub>·2H<sub>2</sub>O, 0.2 gm.; syn-  
thetic iron humate, 5 p.p.m.; H<sub>2</sub>O, 1000 cc. pH 7.4.

Basal medium II: Same as medium I except K<sub>2</sub>HPO<sub>4</sub>, 0.7 gm. and KH<sub>2</sub>PO<sub>4</sub>, 0.3 gm. as phosphate buf-  
fer. pH 6.9.

Light: Artificial, continuous, approximately 350 foot candles.

Incubation period: 44 days (10/28/33-11/11/33).

the CaCO<sub>3</sub> makes it as satisfactory as any yet tested. In the presence of the CaCO<sub>3</sub> the initial growth rate is comparatively rapid, but death usually occurs after a few days without aeration unless the

illumination is greatly reduced. Death is due to a rapid increase in pH, as will be shown later. The addition of a suitable sugar usually greatly improves this medium and allows growth in the presence of  $\text{CaCO}_3$  to continue for a longer period. For instance, table 1 shows a fixation of 9-17 mg. N in 100 cc. of media containing 1 per cent glucose and 0.1 per cent  $\text{CaCO}_3$  during a period of 85 days. At the

TABLE 3  
NITROGEN FIXATION UNDER VARYING LIGHT CONDITIONS AND  
PARTIAL PRESSURES OF CARBON DIOXIDE

ILLUMINATION (MAZDA LAMPS)		MEDIUM USED	AERATED WITH 1% $\text{CO}_2$ IN AIR			AERATED WITH 5% $\text{CO}_2$ IN AIR		
DAILY PERIOD (HOURS)	APPROXI- MATE IN- TENSITY (FOOT CANDLES)		NITRO- GEN FIXED (MG.)	AVERAGE (MG.)	FINAL PH	NITRO- GEN FIXED (MG.)	AVERAGE (MG.)	FINAL PH
24	350	I	18.5	.....	7.2	17.3	.....	7.0
24	"	"	18.0	18.25	7.2	15.2	16.25	6.9
24	175	"	14.5	.....	7.0	11.6	.....	7.1
24	"	"	11.8	13.15	6.9	9.4	10.50	7.0
18	350	"	16.7	.....	7.2	17.9	.....	7.0
18	"	"	13.3	15.00	7.2	17.4	17.65	7.0
24	"	II	6.4	.....	7.2	.....	.....	.....
24	"	"	6.9	6.65	7.3	.....	.....	.....
24	175	"	6.0	.....	7.2	.....	.....	.....
24	"	"	5.0	5.50	7.3	.....	.....	.....
18	350	"	7.2	.....	7.2	.....	.....	.....
18	"	"	6.7	6.95	7.2	.....	.....	.....

Basal medium I:  $\text{K}_2\text{HPO}_4$ , 1.0 gm.;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.2 gm.;  $\text{NaCl}$ , 0.2 gm.;  $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ , 0.1 gm.; synthetic iron humate, 5 p.p.m.;  $\text{CaCO}_3$ , 10 gm.;  $\text{H}_2\text{O}$ , 1000 cc.

Basal medium II: Same as medium I except  $\text{K}_2\text{HPO}_4$ , 0.2 gm. and  $\text{NaHCO}_3$ , 1.0 gm. added.

Incubation period: 81 days (12/21/33-3/12/34).

end of the experiment the cultures were nearly dead, but it is probable that the sugar was practically all utilized before the cultures began to turn yellow.

ENERGY SOURCES.—Sunlight is a satisfactory source of energy for providing the materials needed for respiration and growth, but usually the rate of growth is accelerated by the addition of suitable sugars. An intensive study of the kinds of sugar used by *Nostoc* has not yet been made, but the two tested, sucrose and glucose, have both increased growth in light. In table 2, the nitrogen fixation in

TABLE 4  
GROWTH AND NITROGEN FIXATION AT VARIOUS  
H ION CONCENTRATIONS

INITIAL PH	RELATIVE GROWTH (14 DAYS)	NITROGEN FIXATION (44 DAYS)		FINAL PH
		TOTAL (MG.)	AVERAGE (MG.)	
5.2	None	0 0	0	5.6 5.6
5.5	"	0 0	0	5.8 5.8
5.7	"	0 0.13	0.07	6.0 6.2
6.0	Slight	1.95 1.62	1.79	6.2 6.2
6.3	"	1.65 1.45	1.55	6.3 6.4
6.5	"	1.56 1.23	1.40	6.4 6.4
6.6	"	2.32 1.24	1.78	6.5 6.5
6.8	Fair	1.48 1.51	1.50	6.7 6.7
7.4	"	1.59 1.56	1.58	7.1 7.1
7.6	"	2.34 1.92	2.13	7.4 7.4
8.0	"	1.74 1.89	1.82	7.8 7.8
8.4	"	1.47 2.53	2.00	8.4 8.8

Basal medium:  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.2 gm.;  $\text{NaCl}$ , 0.2 gm.;  $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ , 0.2 gm.; synthetic iron humate, 5 p.p.m.;  $\text{H}_2\text{O}$ , 1000 cc.; potassium phosphates (mono-, di- or tri- to give desired pH), 1.0 gm.

Aeration: Un aerated.

Light: Artificial, continuous, approximately 350 foot candles.

Incubation period: 44 days (10/28/33-11/11/33).



aerated cultures containing no  $\text{CaCO}_3$  was 5.34 mg. N without sugar and 11.80 with sucrose; the corresponding figures with  $\text{CaCO}_3$  were 9.81 and 18.63. Glucose in this experiment, as frequently happens, produced a toxic effect, owing to its decomposition in the autoclave when sterilized in an alkaline medium, even though the sterilization lasted for only 10 minutes at 10 pounds' pressure. In an earlier experiment (table 1), however, good growths were obtained in a some-

TABLE 5  
PHOSPHATE BUFFERS

PHOSPHATE ADDITIONS TO BASAL MEDIUM (GM. PER LITER)		INITIAL PH	NITROGEN FIXED (MG.)	FINAL PH
$\text{KH}_2\text{PO}_4$	$\text{K}_2\text{HPO}_4$			
0.1	.....	7.0	8.85	5.8
0.2	.....	6.6	7.91	5.8
0.5	.....	6.4	1.47	5.8
1.0	.....	6.2	0.22	5.8
2.0	.....	6.0	0.26	5.6
5.0	.....	5.4	0.19	5.1
.....	0.1	7.0	6.70	5.8
.....	0.5	7.4	10.45	5.8
.....	5.0	8.2	3.39	7.3
0.05	0.05	7.0	10.15	5.4
0.25	0.25	6.8	7.71	5.6
2.5	2.5	6.8	3.39	6.6
0.09	0.01	7.1	8.52	5.6
0.45	0.05	6.6	4.31	5.8
4.5	0.5	6.0	0.12	5.6

Basal medium:  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.2 gm.;  $\text{NaCl}$ , 0.2 gm.;  $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ , 0.1 gm.;  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , 0.005 gm.;  $\text{CaCO}_3$ , 0.2 gm.;  $\text{H}_2\text{O}$ , 1000 cc.

Aeration: Aerated with 1%  $\text{CO}_2$  in air.

Light: Artificial, 12-hour day, approximately 300 foot candles.

Incubation period: 66 days (4/22/30-6/27/30).

what similar medium. Separate sterilization of the glucose in a neutral or preferably slightly acid solution is advisable.

NITROGEN SOURCES.—Ammonium nitrate, ammonium sulphate, potassium nitrate, and asparagin have been used in different experiments. The growth with potassium nitrate was usually better than with the other nitrogen sources, probably because it makes the medium more alkaline (table 7). This increase is desirable in cultures aerated with 1 per cent  $\text{CO}_2$  in air, of course, but might be very

TABLE 6  
EFFECT OF IRON, BORON, AND MANGANESE ON NITROGEN FIXATION

TREATMENT	FIRST EXPERIMENT	SECOND EXPERIMENT		
	NITROGEN FIXED (MG.)	NITROGEN FIXED (MG.)	AVERAGE (MG.)	FINAL PH
Check (basal medium without $\text{FeCl}_3$ )..	17.69	6.63 8.73	7.68	7.0 6.9
Check (basal medium).....	17.49	8.17 8.48	8.33	7.3 7.3
Ferric citrate, 2 p.p.m. Fe (no $\text{FeCl}_3$ ).....		9.03 9.42	9.26	7.2 7.3
Ferric citrate, 20 p.p.m. Fe (no $\text{FeCl}_3$ ).....		7.73 7.16	7.45	7.2 7.2
Natural humic acid, 50 p.p.m. (no $\text{FeCl}_3$ ).....		7.30 9.32	8.31	7.0 6.8
Natural humic acid, 50 p.p.m.....		8.19 10.90	9.55	6.7 7.1
Ferric citrate, 2 p.p.m. Fe (no $\text{FeCl}_3$ ) + natural humic acid, 50 p.p.m.....		8.76 8.58	8.67	7.0 7.0
Boric acid, 0.1 p.p.m.....	13.49			
Boric acid, 1 p.p.m.....	13.79	7.16 7.93	7.55	7.4 7.3
Boric acid, 10 p.p.m.....	19.99			
Boric acid, 20 p.p.m.....		8.62 6.49	7.56	7.1 7.0
$\text{MnSO}_4$ , 5 p.p.m.....	14.79	7.54 6.28	6.91	7.0 7.1
$\text{MnSO}_4$ , 50 p.p.m.....	14.29	9.15 8.34	8.75	6.8 6.8
Boric acid, 0.1 p.p.m. + $\text{MnSO}_4$ , 5 p.p.m.....	11.79			

Basal medium:  $\text{K}_2\text{HPO}_4$ , 0.5 gm.;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.2 gm.;  $\text{NaCl}$ , 0.2 gm.;  $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ , 0.1 gm.;  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , 0.005 gm.;  $\text{CaCO}_3$ , 1.0 gm.;  $\text{H}_2\text{O}$ , 1000 cc. (first experiment 200 cc. medium per flask; second experiment 100 cc.).

Aeration: Aerated with 1%  $\text{CO}_2$  in air.

Light: Artificial, 12-hour day, approximately 300 foot candles.

Incubation period: First experiment 56 days (4/2/30-5/28/30); second 55 days (6/5/30-7/30/30).

undesirable in unaerated cultures. Ammonium nitrate and ammonium sulphate make the medium sufficiently acid to retard growth or even to kill the culture unless adequate  $\text{CaCO}_3$  or other buffer is present.

TABLE 7

## IMPORTANCE OF CALCIUM AND STRONTIUM IN NITROGEN FIXATION

TREATMENT (ADDITIONS PER 100 CC. MEDIUM)	FIRST EXPERIMENT*		SECOND EXPERIMENT*		THIRD EXPERIMENT*†	
	NITROGEN IN CELLS (MG.)	FINAL PH	TOTAL NITROGEN (MG.)	FINAL PH	TOTAL NITROGEN (MG.)	FINAL PH
Check (basal medium only).	0.87	7.0	1.51	6.7	3.10	6.7
$\text{KNO}_3$ , 10 mg. N.....	.....	.....	.....	.....	8.80†	7.3
$\text{KNO}_3$ , 20 mg. N.....	5.29	7.8	12.81†	7.6	.....	.....
$\text{CaSO}_4$ , 10 mg.....	.....	.....	2.73	6.7	3.67	6.7
$\text{CaSO}_4$ , 20 mg.....	1.32	6.4	.....	.....	.....	.....
$\text{CaSO}_4$ , 50 mg.....	.....	.....	1.15	6.2	1.26	6.1
$\text{CaSO}_4$ , 20 mg.+ $\text{KNO}_3$ , 20 mg. N.....	4.51	7.8	.....	.....	.....	.....
$\text{SrSO}_4$ , 20 mg.....	1.89	7.0	2.18	6.7	4.32	6.7
$\text{SrSO}_4$ , 20 mg.+ $\text{KNO}_3$ , 20 mg. N.....	4.90	7.9	.....	.....	.....	.....
$\text{CaCO}_3$ , 14.7 mg.....	1.88	7.4	.....	.....	.....	.....
$\text{CaCO}_3$ , 36.8 mg.....	.....	.....	2.57	7.1	5.43	7.0
$\text{CaCO}_3$ , 14.7 mg.+ $\text{KNO}_3$ , 20 mg. N.....	8.19	8.1	.....	.....	.....	.....
$\text{CaSO}_4$ , 20 mg.+ $\text{CaCO}_3$ , 14.7 mg.....	1.45	7.2	.....	.....	.....	.....
$\text{CaSO}_4$ , 20 mg.+ $\text{CaCO}_3$ , 14.7 mg.+ $\text{KNO}_3$ , 20 mg. N.....	6.57	7.9	.....	.....	.....	.....
$\text{CaSO}_4$ , 20 mg.+ $\text{CaCO}_3$ , 14.7 mg.+asparagin, 20 mg. N.....	4.19	7.0	.....	.....	.....	.....

\* All results are averages of two or more cultures.

† Inoculated with culture grown on calcium-free medium.

‡ Nitrogen in cells only.

Basal medium:  $\text{K}_2\text{HPO}_4$ , 0.75 gm.;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.2 gm.;  $\text{NaCl}$ , 0.2 gm.;  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , 0.005 gm.;  $\text{H}_2\text{O}$ , 1000 cc.

Aeration: Aerated with 1%  $\text{CO}_2$  in air.

Light: Artificial, 12-hour day, approximately 300 foot candles.

Incubation period: First experiment 23 days (4/2/30-4/25/30); second 47 days (6/18/30-8/4/30); third 53 days (8/11/30-10/3/30).

## ILLUMINATION

The optimum light intensity was found to depend to a marked extent upon the growth conditions, particularly the medium used, amount of  $\text{CO}_2$  or sugar available, and possibly upon the degree of

TABLE 8  
IMPORTANCE OF CALCIUM IN NITROGEN FIXATION

TREATMENT (ADDITIONS PER 100 CC. MEDIUM)	TOTAL NITROGEN (MG.)	AVERAGE (MG.)	FINAL PH
Check (basal medium only).....	0.13 0.10	0.12	6.9 6.9
CaSO <sub>4</sub> , 20 mg.....	0.42 0.45	0.44	6.7 6.7
MgSO <sub>4</sub> , 20 mg.....	0.54 0.13	0.34	6.9 6.9
CaSO <sub>4</sub> , 20 mg.+MgSO <sub>4</sub> , 20 mg....	2.95 2.19	2.57	6.6 6.6
KNO <sub>3</sub> , 10 mg. N.....	2.05* 0.46*	1.26*	6.9 6.8
KNO <sub>3</sub> , 10 mg. N+MgSO <sub>4</sub> , 2.5 mg...	4.44* 4.01*	4.23*	7.2 7.2
KNO <sub>3</sub> , 10 mg. N+MgSO <sub>4</sub> , 20 mg....	6.29* 0.87*	6.20* 0.87*	7.2 6.9
KNO <sub>3</sub> , 10 mg. N+MgSO <sub>4</sub> , 100 mg...			

\* Nitrogen in cells only.

Basal medium: K<sub>2</sub>HPO<sub>4</sub>, 0.75 gm.; H<sub>2</sub>O, 1000 cc.

Aeration: Aerated with 1% CO<sub>2</sub> in air.

Light: Artificial light, 12-hour day, approximately 300 foot candles.

Incubation period: 53 days (8/11/30-10/3/30).

Inoculum: Used culture grown on calcium-free medium consisting of K<sub>2</sub>HPO<sub>4</sub>, 0.75 gm.; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.2 gm.; H<sub>2</sub>O, 1000 cc.

TABLE 9  
NITROGEN FIXATION IN THE DARK IN UNAERATED MEDIA

	FIRST EXPERIMENT (98 DAYS)	SECOND EXPERIMENT (188 DAYS)
Number of cultures.....	15	21
Average nitrogen fixed per 100 cc.....	2.6 mg.	2.5 mg.
Average glucose consumed.....	290.0 "	250.0 "
Average efficiency (N fixed per gm. glucose consumed)...	9.1 "	10.2 "
Maximum fixation per 100 cc.....	5.4 "	5.6 "
Maximum efficiency.....	14.8 "	13.2 "

Basal medium: K<sub>2</sub>HPO<sub>4</sub>, 0.5 gm.; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.2 gm.; NaCl, 0.2 gm.; CaSO<sub>4</sub>·2H<sub>2</sub>O, 0.1 gm.; FeCl<sub>3</sub>·6H<sub>2</sub>O, 0.005 gm.; glucose, 10 gm.; H<sub>2</sub>O, 1000 cc. Additional buffers (CaCO<sub>3</sub>, K<sub>2</sub>HPO<sub>4</sub>, or K<sub>3</sub>PO<sub>4</sub>) supplied in a few flasks for adequate control of pH.

aeration. Under most and possibly all conditions direct sunlight seems to be too intense an illumination for best growth, although good growths and nitrogen fixation have been obtained when cultures were exposed to direct sunlight in the laboratory window for four-six hours daily. A considerably lower light intensity is certainly preferable. The diffuse light of an ordinary well lighted room is very satisfactory for the maintenance of cultures in a healthy condition. The writers have kept a few liquid cultures in cotton-stoppered flasks under such conditions for about six years without transfer to fresh media, and they are still in remarkably good condition.

The light intensities chosen for use in these investigations, after several preliminary experiments, ranged from 175 to 350 foot candles, usually near 300 foot candles. Even this intensity sometimes seemed too high, and hence the experiment reported in table 3 was carried out. It will be observed that in the phosphate medium continuous light of an intensity of 175 foot candles in comparison with 350 foot candles produced on the average about 68 per cent as large a nitrogen fixation. Observations during growth, however, showed that the initial growth (where 1 per cent  $\text{CO}_2$  was used) was much more rapid at the higher light intensity. These cultures showed a tendency to turn slightly yellow after a few weeks, while the cultures in the lower light remained green. With 5 per cent  $\text{CO}_2$  the growth rate was slower, especially at first, as will be explained in a later section.

There were certain indications throughout these investigations that the alga needs a daily rest period of non-illumination, and that a 12- to 18-hour daily period of illumination is preferable to continuous light. The data of table 3 report a direct experiment to study this point. It may be seen that there were no very marked differences in the final results between an 18- and a 24-hour day. The 18-hour cultures seemed to be slightly better than the others maintained at the same light intensity, but not better than those under continuous light at the lower light intensity. Evidently these organisms do not require a rest period if the light intensity is sufficiently low and the growth conditions are satisfactory. The favorable effect of the rest period, sometimes observed, is probably due in large measure to the decrease in total daily illumination. In work with *Chlorella* a 12-hour

day is commonly used, and probably a similar daily period of illumination for *Nostoc* might prove most satisfactory for most experiments, especially when continued for several weeks.

Since the light from a Mazda lamp contains a much higher percentage of long rays than does sunlight, tests were made to determine whether the elimination of these would affect growth favorably. When glass filters which removed the infra red and a considerable portion of the visible red rays were used, a somewhat slower growth rate resulted, due in all probability to the reduction in total illumination. The filters were therefore not used in later experiments.

#### CARBON DIOXIDE SUPPLY

Un-aerated cultures, containing no sugar in the medium, make a relatively poor growth, especially if air is the only source of  $\text{CO}_2$ . This slower growth of un-aerated cultures, assuming that the pH remains satisfactory, is due chiefly to lack of  $\text{CO}_2$ , since the addition of glucose to such un-aerated cultures will permit good growth. Qualitative observations indicate that the oxygen supply may also be inadequate for maximum growth in un-aerated cultures, but further experimentation would be necessary to establish this point. In most of the experiments a cylinder of compressed air containing 1 per cent  $\text{CO}_2$  was used for aeration. In table 3 are reported comparative nitrogen fixations using 1 and 5 per cent  $\text{CO}_2$ . Generally speaking, 1 per cent proved more satisfactory, chiefly because of the more favorable pH of the medium. Under these experimental conditions the initial pH of the cultures receiving 5 per cent  $\text{CO}_2$  was usually about 0.3 to 0.5 pH lower than when 1 per cent  $\text{CO}_2$  was used.

Observations during the growth of the cultures (table 3) showed that during the first three or four weeks most of the cultures aerated with 5 per cent  $\text{CO}_2$  made very little growth, whereas with the lower partial pressures of  $\text{CO}_2$  the growth was excellent. During the second month there was a marked improvement in the cultures receiving 5 per cent  $\text{CO}_2$ , and at the time of analysis they were about as good as those receiving 1 per cent  $\text{CO}_2$ . Since the difficulty in keeping a culture at pH 7 or above increases with  $\text{CO}_2$  concentration, there would seem to be no adequate reason for using air containing more than 1 per cent  $\text{CO}_2$ .

## HYDROGEN ION CONCENTRATION

LIMITS OF GROWTH.—A neutral or alkaline medium is decidedly preferable for this species of *Nostoc*. Table 4 shows that growth does not take place below a pH of approximately 5.7. This figure has been checked in aerated and unaerated cultures and invariably the lower limit of growth initiation has been between 5.7 and 5.9. Likewise, when growing cultures show signs of death because of increasing H ion concentration, the pH observed at the first signs of yellowing is usually between 5.5 and 5.7, slightly lower than the limit of growth initiation, as would be expected. No study has yet been made to determine whether *Nostoc* can grow at a considerably lower pH if given combined nitrogen, as BURK (10) observed in the case of *Azotobacter*.

Table 4 also shows that normal growth occurs at a pH of 8.4 to 8.8. The exact upper limit for growth has not yet been determined but apparently it is at a pH of approximately 9. As will be shown later in figure 9, if the alga is grown in unaerated media in the presence of carbonates the pH may rise to 9.4–9.6, killing practically all vegetative cells.

OPTIMUM PH.—Growth is usually best at the pH range of about 7.0 to 8.5, and decreases in going from pH 7 to 6, especially toward the lower limit. This is not shown by the results in table 4 because these were unaerated cultures, but is based upon the general observations made during the course of these studies. When the pH was such as to allow growth to be initiated, then at the end of 44 days the fixations were nearly the same in all flasks, but the nitrogen fixed was small (table 4). If an adequate supply of CO<sub>2</sub> had been available, such uniform fixations would not have been obtained.

BUFFER SALTS.—The buffer used in most of the experiments was K<sub>2</sub>HPO<sub>4</sub>. In a synthetic medium such as recommended on a previous page, but containing no carbonate, the K<sub>2</sub>HPO<sub>4</sub> gives a pH of about 7.2–7.4, which is optimum. Aeration with 1 per cent CO<sub>2</sub> reduces the pH to about 7.0. If 0.1 to 1.0 per cent CaCO<sub>3</sub> is present, however, the pH is usually maintained at about 7.2 to 7.5 when so aerated.

Table 5 gives the results of a study of different proportions and quantities of mono- and di-potassium phosphate buffers. Too much

emphasis should not be placed upon the final pH values, since older cultures, containing dead cells, frequently show a low pH. The greatest nitrogen fixation was obtained with 0.05 per cent  $K_2HPO_4$ , while a concentration of 0.5 per cent was somewhat toxic. In other experiments there were some indications that even a concentration of 0.1 per cent may be above the optimum.

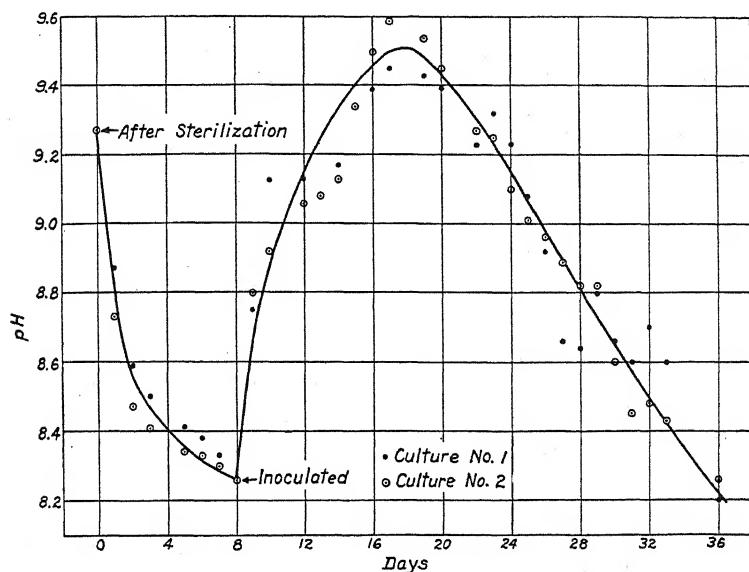


FIG. 9.—Effect on pH caused by addition of heavy inoculum of *Nostoc muscorum* to unaerated inorganic medium containing 1 per cent  $CaCO_3$  and exposed to continuous light.

Other buffers, such as  $NaHCO_3$  (table 3) and  $Na_2HPO_4$ , were used in a few experiments but with less satisfactory results, at least at the concentrations chosen.  $NaHCO_3$  cannot be used satisfactorily in unaerated culture media because of the marked rise in pH, similar to that which occurs with  $CaCO_3$ .

**CARBONATES AND PH.**—Figure 9 gives results showing the rise in pH brought about by algal cultures growing in an inorganic medium containing 1 per cent  $CaCO_3$  and unaerated. The H ion determinations were made by means of the glass electrode, using Ag-AgCl against HgCl-Hg with saturated KCl as the conducting bridge. The



culture vessels (500 cc. balloon flasks) contained 100 cc. portions of the sterile medium with the sterile glass electrodes immersed in it. Continuous light of about 400 foot candles intensity was used and the temperature maintained at  $27^{\circ}$ – $31^{\circ}$ C. After eight days, when the pH of the medium had become fairly constant, heavy inocula of the algal mass were added; otherwise the flasks were left undisturbed throughout the experiment. The daily readings of duplicate cultures are given in figure 9, with the curve drawn so as to represent the average result.

The freshly autoclaved medium had a pH of 9.27, owing undoubtedly to the driving off of  $\text{CO}_2$  through heating. After standing undisturbed for eight days in contact with air the pH had dropped to 8.26. As soon as the heavy inocula of the alga were added the pH began to rise rapidly, reaching a maximum of 9.45–9.59 on the ninth day subsequent to inoculation. This killed all or practically all of the actively growing cells and hence the pH then began a gradual decline. Nineteen days later it was back at approximately the same point as when inoculated. In other similar experiments, where small inocula were used, the rise in pH to the death point under continuous illumination required about three weeks.

Figure 9 shows that it is the removal of  $\text{CO}_2$  from  $\text{CaCO}_3$  by the actively photosynthesizing culture, with the resulting formation of the hydroxides of calcium and the other basic elements present, that results in the rise in pH. It is perhaps surprising that the alga will continue to photosynthesize up to the point where the resulting high alkalinity causes death. The pH of algal cultures containing dead cells was somewhat slower in returning to the original value, 8.2, than that of the freshly sterilized medium. This was probably due primarily to the fact that a few algal cells were not killed and were able to grow slowly when the alkalinity had decreased sufficiently. The very slow recovery of cultures which earlier appeared to be dead from high pH has been observed frequently.

#### TEMPERATURE

The temperature of  $24^{\circ}$ – $27^{\circ}$ C. used in most of the experimental work reported here was satisfactory. The optimum has not been determined accurately, but over the range of  $25^{\circ}$ – $30^{\circ}$ C. no great difference

in the rate of growth was observed. At higher temperatures, especially at  $35^{\circ}$ – $40^{\circ}$  C., the growth rate was much slower and the cells, when observed under the microscope, frequently appeared to be abnormal. At the lower temperatures ( $5^{\circ}$ – $10^{\circ}$  C.) the vegetative cells commonly go into a resting condition, in which they will remain dormant for several months. When taken out of the refrigerator active cell division begins within about a week. These resting filaments are not true spores, but resemble ordinary vegetative filaments except that the cells become somewhat larger and distinctly egg-shaped.

#### EFFECT OF SOIL HUMIC ACID

Natural humic acid frequently acts as a growth stimulant for a variety of plant types, including both higher green plants and microorganisms. Recent work (2, 10, 11, 12, 21), carried out in this laboratory, has shown that the cause of this stimulation may vary for different types of plants, and even for the same plant grown in different media. In the case of higher plants the stimulation is usually due chiefly to the available iron added. This humic acid iron does not precipitate readily, even in an alkaline medium, which is in distinct contrast to the behavior of inorganic iron salts. Certain trace elements, present in the humic acid, may also often be responsible in part for the increased growth of higher plants. In the case of *Azotobacter* the humic acid effect is usually due to the addition of available iron, molybdenum, or vanadium. Certain strains of rhizobia frequently show an exceptionally large growth increase following additions of natural humic acid. This was shown (2) to be due chiefly to the presence of "coenzyme R," an essential growth substance required by these bacteria.

The results of studies to determine the response of *Nostoc* to natural humic acid are given in tables 1 and 6. The humic acid was prepared by the extraction of a fertile soil with alkali, followed by precipitation with acid and re-solution with alkali, according to the usual methods.

Nitrogen fixation was increased by the highest rate of application (table 1) from 9.07 to 17.28 mg. N per 100 cc. This large increase occurred in an unaerated medium containing  $\text{CaCO}_3$  and having an

initial pH of 7.8. The results of the second experiment (table 1) agree with those of the first, even though these cultures were aerated with 1 per cent  $\text{CO}_2$  and the pH was only slightly alkaline. In table 6, however, the effect of humic acid, as well as of ferric chloride and ferric citrate, was small. In the two experiments where humic acid gave the greatest effect the media contained sugar, while in the other case no sugar was present. The incubation periods were longer and the total growth likewise larger in the presence of the sugar. No attempt has yet been made to determine the primary cause of the humic acid stimulation, but it seems probable that the available iron is of greatest importance.

#### TESTS WITH BORON AND MANGANESE

In table 6 are given the results of tests of boron and manganese on growth and nitrogen fixation. There was no indication that either of these elements is needed in quantities greater than those found as impurities in the medium.

#### CALCIUM AND STRONTIUM IN NITROGEN FIXATION

It was recently reported from this laboratory (13) that either calcium or strontium is essential for nitrogen fixation by *Azo. vinelandii*, but not for growth in the presence of an adequate supply of fixed nitrogen. Similar tests with *Nostoc* are reported in table 7. No attempt was made to free the basal medium of the last traces of calcium and strontium.

The results of the first experiment reported in table 7 would seem to indicate that neither calcium nor strontium, at least in concentrations greater than the traces present as impurities, is needed for growth. Some nitrogen fixation also occurred in the absence of added calcium or strontium, but at a considerably reduced rate. The effects of  $\text{CaSO}_4$ ,  $\text{CaCO}_3$ , and  $\text{SrSO}_4$  on nitrogen fixation were essentially the same, but  $\text{CaCO}_3$  produced an appreciably better growth in the presence of fixed nitrogen than did the other compounds. The results of the second and third experiments (table 7) agree essentially with those of the first.

In further studies, reported in table 8, a basal medium consisting of only  $\text{K}_2\text{HPO}_4$  was used and the inoculating culture was again

grown on a calcium-free medium. These data show that magnesium is essential for both growth and nitrogen fixation, as would be expected since this element is a constituent of chlorophyll. In the medium containing  $K_2HPO_4$  and  $MgSO_4$  practically no growth was obtained, except where either combined nitrogen or  $CaSO_4$  was added. The small growth obtained under these conditions, corresponding to 0.22 mg. N, can be accounted for on the basis of the calcium impurity in the  $MgSO_4$ . The addition of  $CaSO_4$  to this medium brought about normal growth and a fixation of nitrogen amounting to 2.57 mg. N, or approximately half as good a growth as with  $KNO_3$  and no calcium. Although no flasks were included in this particular experiment with both  $CaSO_4$  and  $KNO_3$  present, it may be stated fairly definitely from other experiments that the growth under these conditions would not have been more than 50 per cent greater than with the nitrate in the absence of calcium.

These data suggest that calcium is essential for nitrogen fixation but probably not for growth. In the case of the results reported in table 7, it is probable that the traces of calcium present in the medium were adequate for the fixation obtained.

In another similar experiment, except that  $NaHCO_3$  was used as a buffer instead of  $K_2HPO_4$ , the necessity for a supply of phosphorus for growth was shown conclusively.

#### NITROGEN FIXATION IN THE DARK

*Nostoc muscorum* will live and grow for months in the dark and form normal appearing chlorophyll if given a suitable energy source, such as glucose. Growth and nitrogen fixation take place rather slowly but the quantities fixed per unit of energy consumed are usually large. Table 9 gives a summary of the results of two experiments carried out in the dark except for brief growth observations at intervals of about one month. In these experiments a basal inorganic salts medium containing 1 per cent glucose was used.  $CaCO_3$  was added to a few flasks and additional phosphate was supplied to others in order to increase the buffering capacities. Several of the cultures not supplied with  $CaCO_3$  were nearly dead when analyzed, owing to the increase in acidity, the pH in these cases usually being 5.4–5.8. This increase was undoubtedly due to organic acids formed,

since growth in the presence of  $\text{CaCO}_3$  continued normally throughout the incubation period. In these flasks the final pH was near 7 and the largest fixations and usually the highest efficiencies were obtained. An average fixation of 9.6 mg. N per gram of glucose consumed, with a maximum value of 14.8 mg. N under ordinary atmospheric conditions of growth, is higher than commonly obtained with *Azotobacter* or other nitrogen-fixing bacteria.

#### GROWTH CYCLE AS FACTOR IN PHYSIOLOGICAL STUDIES

These studies, considered as a whole, emphasize the difficulty sometimes experienced in securing close agreements between experiments carried out from time to time under similar but not necessarily exactly identical experimental conditions. Considerable time was spent in determining the causes for these growth variations. Aside from the numerous points already considered, such as pH,  $\text{CO}_2$  concentration, and light intensity, the growth cycle of the organism should not be overlooked. In the case of bacteria, where growth consists largely of cell enlargement followed by cell division, the process is relatively simple. In *Nostoc* the growth cycle may vary from time to time. Perhaps the most common cycle is for resting vegetative cells, upon renewing their activity, to divide, thus increasing the length of the trichomes. After a growth period of two to five days these cells commonly break at the heterocysts or separation discs, forming young hormogones which are actively motile for a few hours, during which time they become long and slender. They may then come to rest, the cells increase about twofold in diameter, and heterocyst formation occur. Cell division of these trichomes may or may not continue uninterrupted. These trichomes may again form new hormogones, they may come to rest and remain so for several days, or they may break up into jelly-like masses consisting of the individual cells held together by the gel and showing little resemblance to the original filamentous arrangement. These single cells may in turn divide and become new hormogones. Under other conditions the organism may go into the spore stage, frequently practically all vegetative cells showing such a change. These spores may, of course, develop into new trichomes. As a result of these and other possibilities, cultures when examined under the microscope

may show marked differences in appearance from day to day, and it is not surprising that growth rates may vary markedly. The appearance of the organism in older cultures in contrast to its appearance when young is especially marked. This suggests that most quantitative work done with this organism with a view to establishing functional relations might profitably be limited to much shorter incubation periods than used in most of the experiments reported here.

#### IMPORTANCE OF BLUE-GREEN ALGAE IN SOIL

Practically all cultivated soils and grasslands have a rather abundant flora of blue-green algae, various species of *Nostoc* being especially common. In fact the majority of the members of this genus are terrestrial. BRISTOL (7), MOORE and CARTER (28), and LOWE and MOYSE (23) reported the finding of *Nostoc muscorum* in soils. Additional studies dealing with the occurrence of blue-green algae in soils were reported by ESMARCH (16, 17), ROBBINS (31), BRISTOL (8, 9), and PETERSEN (29). RUSSELL (32) emphasizes the importance of algae, especially blue-greens, in the colonization of new ground following volcanic eruptions. PETERSEN (29) points out that the limited data available at present indicate that a gram of soil usually contains about 10,000 to 100,000 algae (greens and blue-greens). Calculations of the actual mass of algal cells in soil show that their weight is often almost as great as that of the bacteria.

Considerable work has been reported in which attempts were made to determine whether algae can fix free nitrogen. MOORE and CARTER (28), DREWES (15), JONES (22), and PETERSEN (29) have reviewed the literature. It has been shown repeatedly that substrates frequently show gains in nitrogen where algal growths are abundant. Some of the earlier workers, such as BEIJERINCK (5) in 1901, were convinced that blue-green algae can utilize free nitrogen gas, but they did not work with bacteria-free cultures. Likewise MOORE and WEBSTER (26), and MOORE, WHITLEY, and WEBSTER (27) held similar views but did not prove them. Attempts at proof, using pure cultures, including a *Nostoc* sp., were made by PRINGSHEIM (30), GLADE (20), and MAERTENS (24) with negative results. The view then became rather prevalent that none of the algae can utilize nitrogen gas. Even as late as 1930, JONES (22) concluded

from work with three species of the Cyanophyceae that the algae live in association with nitrogen-fixing bacteria to the mutual benefit of both organisms. Work in this laboratory (4) with eight cultures of the Cyanophyceae, obtained from PRINGSHEIM, showed no nitrogen fixation. The work of MOLISCH (25), VOUK and WELLISCH (34), DREWES (15), and COPELAND (14), previously mentioned, as well as the results obtained at this laboratory (1, 3, 4) with *Nostoc muscorum*, stand out in contrast to these negative results. Evidently the ability to fix nitrogen is not a universal characteristic of all the Cyanophyceae but is probably limited to a few genera and species.

One of the most striking points brought out by the researches reported here, as well as in earlier publications from this laboratory, is the comparatively large quantities of nitrogen fixed by *Nostoc muscorum* in a given length of time. Fixations of 10 to 15 mg. N per 100 cc. of inorganic culture medium, aerated with air containing CO<sub>2</sub>, were frequently obtained during an incubation period of two to three months, starting with a small inoculum. This is a ten- to twenty-fold greater fixation than reported for other blue-green species by the investigators just mentioned. In old cultures, at least, a considerable portion of this nitrogen was found (4) to be present in a soluble form. In the dark, nitrogen fixation proceeds slowly but the food supply is utilized comparatively efficiently.

A summation of our knowledge of the occurrence and role of blue-green algae in nature would seem to indicate that these organisms are of considerable importance in the maintenance of soil fertility. A similar conclusion was reached by BRISTOL (8), even though the nitrogen-fixing ability of certain Cyanophyceae had not been demonstrated conclusively at that time. PETERSEN (29) is doubtful as to the economic importance of algae in soil, basing his views largely on the supposition that they make little growth except at the soil surface. He considers that the Cyanophyceae, with the exception of *Nostoc punctiforme* and possibly a few others, cannot grow heterotrophically or fix nitrogen in the dark. Our results with the most active nitrogen-fixing blue-green alga yet isolated from soil, together with the recent results of WINTER (35) with *Nostoc punctiforme*, definitely contradict these two ideas which serve in large part as a basis for PETERSEN's viewpoint. Whether *Nostoc muscorum* actually

makes an appreciable growth in soil where light does not penetrate remains to be determined, but it is at least of interest to know that it has the ability to do so.

If the results reported here with the one species are typical, it would seem that the nitrogen-fixing blue-greens thrive best in nearly neutral or alkaline soils, preferably partly shaded, and where moisture is abundant. *Nostoc* also grows abundantly in fresh water. These studies suggest that even in acid soils it may be able to continue to multiply at the surface, because by growing in colonies and constantly removing  $\text{CO}_2$  from the soil during photosynthesis it may increase the pH locally. Its gelatinous covering also enables the organism to withstand remarkably dry soil conditions, as FRITSCH (18) and others have pointed out.

The nitrogen-fixing algae, growing near the soil surface, are unique in being able to obtain both their carbon and nitrogen from the air. This, of course, explains why they appear so soon on new volcanic soils and in other places where the soil is too poor to support most other forms of plant life. The quantity of organic matter added as a result of such growth is small but may be of considerable importance in the improvement of such soils.

The algae, like other organisms, compete with higher plants for mineral foods. This competition, while possibly harmful at times, is more likely to have an over-all beneficial effect through the prevention of leaching and the keeping of a reserve supply of mineral elements in a semi-available form for the use of higher plants.

We know comparatively little about the various other chemical transformations which the Cyanophyceae may bring about, but the evidence indicates strongly that per gram of weight they are far less active than common soil bacteria and fungi.

Our information concerning the nitrogen-fixing endophytic blue-green algae is still too limited to permit judgment as to their value in adding to nature's supply of combined nitrogen.

### Summary

1. These pure culture studies show the conditions most favorable for growth and nitrogen fixation by the common blue-green alga, *Nostoc muscorum* Ag., previously isolated from soil, freed from other



organisms, and its nitrogen-fixing ability demonstrated. A detailed morphological description of the organism is given.

2. The organism readily obtains both its nitrogen and carbon from the air. Its rate of growth and nitrogen fixation is ten- to twenty-fold greater than that previously reported for other nitrogen-fixing blue-green algae. The quantity of nitrogen fixed is as high as 10 mg. N in 45 days and 18 mg. N in 85 days per 100 cc. of a medium containing no carbohydrate.

3. When kept in the dark in a medium containing glucose, normal appearing chlorophyll forms and growth and nitrogen fixation proceed slowly. A fixation of 10 to 12 mg. N per gram of glucose consumed is common under these conditions.

4. The organism thrives in a variety of media, provided the pH is satisfactory. The lower and upper limits of growth are approximately 5.7 and 9.0, respectively, while the optimum covers the range of about 7.0 to 8.5.

5. A medium containing the usual inorganic salts, without carbonates and sugar, and with  $K_2HPO_4$  as a buffer, is satisfactory for use in unaerated cultures. Growth and nitrogen fixation are much more rapid if the medium is aerated, preferably with air containing about 1 per cent  $CO_2$ . The addition of sugars increases the rate of growth of both aerated and unaerated cultures, especially the latter; likewise combined nitrogen, such as nitrates, ammonia, and asparagin, is usually used somewhat more rapidly than is free nitrogen gas.

6. In unaerated liquid cultures containing an abundance of carbonates and exposed to a high light intensity, the pH rises to about 9.6 and most of the cells die. This results from the utilization of the carbonates as a source of  $CO_2$  when neither adequate  $CO_2$  gas nor sugar is supplied.

7. A light intensity of 175 to 350 foot candles from Mazda lamps gives satisfactory results. A 12-hour day is somewhat preferable to continuous illumination except at the lower light intensities.

8. Neither calcium nor strontium, at least in concentrations greater than traces, is necessary for growth in the presence of combined nitrogen. In a nitrogen-free medium nitrogen fixation markedly decreases in their absence, however, suggesting that they play a cata-

lytic role in nitrogen fixation, as in the case of *Azotobacter*. Natural humic acid increases growth and nitrogen fixation, probably due chiefly to its content of available iron. Boron and manganese have no appreciable beneficial effect.

9. These studies indicate that *Nostoc muscorum* is an organism of considerable importance in soils and possibly in fresh ponds and lakes, adding both nitrogen and organic matter to its growth medium. A brief general discussion of the economic importance of the Myxophyceae in soils is given.

The writers are greatly indebted to Dr. FRANCIS DROUET of the University of Missouri for cooperation in the morphological studies leading to the identification of the organism used, to Dr. R. T. MILNER of this laboratory for assistance in obtaining the data reported in figure 9, and to Mrs. ELLEN K. RIST of this laboratory for making a portion of the nitrogen analyses.

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# NUTRITIONAL LEVELS IN THE PEANUT PLANT

## CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 477

RUFUS H. MOORE

(WITH TWO FIGURES)

### Introduction

PETTIT (12) has discussed the morphology, growth responses, and reproduction of the peanut plant. WALDRON (23) added information concerning the general morphology and physiological response. In both papers the plant is considered indigenous to Brazil, and other species of the Leguminosae which form at least part of their fruits below the surface of the soil are described. REED (17) emphasized the developmental morphology of the fibrovascular bundle, gametophyte, and embryo. Recently STOKES and HULL (19) and HUSTED (4) have reported upon the genetics and cytology of cultivated varieties.

### Investigation

The present investigation concerns the responses of the peanut plant to the variations in the supply of light and available nitrogen it may receive. More specifically the planes of nutrition were varied in order to produce definite type responses as follows: (1) complete suppression of reproductive activity, induced by an abundant supply of readily available nitrogen and a very limited accumulation of carbohydrates; (2) production of flowers and gynophores, but not of fruits, induced by an abundant supply of less readily available nitrogen and a slightly increased accumulation of carbohydrates; (3) an abundance of flowers, gynophores, and fruits, induced by a reduction in the supply of available nitrogen and an increased accumulation of carbohydrates; (4) production of flowers and gynophores but not of fruits, induced by a much restricted supply of available nitrogen and a further accumulation of carbohydrates; and (5) complete suppression of reproductive response, induced by still greater restriction of available nitrogen and a still greater accumulation of carbohydrates.

Essentially these five type responses were secured in the cultural treatments summarized in table 1.

Seed of the Spanish variety, strain 18-38-M-9, selected for thirteen successive generations by the United States Department of Agriculture, was secured. To insure freedom from disease organisms and nodule-forming bacteria, bright and unbroken pods were treated with a 0.25 per cent solution of Uspulun for three successive half-hour periods. As soon as the pods were sensibly dry the seeds were shelled. Seeds were used only from pods which had remained dry inside during the treatment.

TABLE 1  
TYPE RESPONSES IN RELATION TO CULTURAL TREATMENTS

TYPE RESPONSE	CULTURAL TREATMENTS			
	SERIES	SOURCE OF NITROGEN	LENGTH OF DAY	NUMBER OF PLANTS
1.....	A	NH <sub>4</sub>	6 hours	48
2.....	AA	NH <sub>4</sub>	Seasonal	12
2.....	B	NO <sub>3</sub>	8 hours	48
3.....	C	NO <sub>3</sub> and NH <sub>4</sub>	Seasonal	48
4.....	D	NO <sub>3</sub>	Seasonal	48
5.....	E	None	Seasonal	48

NUTRIENT SOLUTIONS.—Certain modifications of the more or less standard nutrient solutions were necessary or useful. Seedling peanut plants were found to require a minimum concentration of calcium salts of 0.0006 gm. mols per liter.<sup>1</sup> Healthier and more vigorous seedlings were produced by using ammonium nitrogen than nitrate nitrogen (9). Concentrations of the phosphate ion ordinarily used in nutrient solutions were found to be injurious to the plant when grown in the light; those grown in darkness are not subject to phosphate toxicity. It was found possible to apply the ammonium nitrogen nutrient solution from a single siphon in drip-culture by reducing the concentrations of the calcium and phosphate ions.

GENERAL REPRODUCTIVE BEHAVIOR.—Under the conditions of light and temperature prevailing in Chicago during the spring, the

<sup>1</sup> Reference to the concentrations of salts in nutrient solutions will always be made on this basis.

peanut plant begins to bloom from four to six weeks after planting. The pre-blooming stage can be extended a week or more by the use of ammonium nitrogen.

From one to eight sessile flowers form in the axil of each leaf. These usually open early in the morning, pollination occurs, and the petals generally begin to wither before the day is over. Thus it is possible to make accurate counts of the number of flowers produced. After several days, the fruit stalk or gynophore begins to elongate noticeably, the dried remains of the floral envelope and included organs often capping the ovary. Elongation of the gynophore is initiated by cells at the base of the ovary (12). The meristem formed here persists until the ovary is pushed a few centimeters below the surface of the soil, or in case the substratum is not reached, until the gynophore is 10 to 15 cm. in length. During the growth of the gynophore the ovary does not increase perceptibly in size. Usually it does not begin to enlarge until it has been buried in the substratum.

The period between the opening of a flower and the beginning of elongation of its gynophore will be referred to as the flower-to-gynophore interval. Data for plants grown in 1933 are summarized in table 2.

RESPONSES TO PHOTOPERIOD AND AVAILABLE NITROGEN.—The peanut, like the tomato, was found to be an indeterminate bloomer. A jar of six plants blossomed abundantly when continuously illuminated for several weeks. Other plants subjected to three- or four-hour periods of daylight under a muslin canopy blossomed sparingly, until approaching exhaustion of their carbohydrates induced progressive death of leaves.

Responses to available nitrogen were studied in 1933 and 1935. In 1933 four series of plants were grown without shading (table 2). Although the concentration of  $\text{Ca}(\text{NO}_3)_2$  supplied to the several series varied over a wide range, all plants were fruitful. Vegetative extension and the size of the crop of fruit were directly correlated with the available nitrogen given. Chemical analyses were made on oven-dried tissues (6, 7) of only the highest and lowest nitrogen series. Chemical data are of some interest (table 3), as the plants were dissected into a greater number of fractions than they were for the analyses of 1935 (tables 6-9).

## CULTURE OF PLANTS FOR MAIN PROBLEM

To grow plants representing the five type responses chosen, the following experiments were conducted in 1935.

All plants received the same treatment from the time of seeding until the specific cultural treatments were begun. On April 12, treated seeds were sown thickly in white quartz sand which had been put into glazed 3-gallon jars, washed with an acid solution, flushed

TABLE 2  
FLOWER-TO-GYNOPHORE INTERVAL (1933)

SERIES	CONCENTRA- TION OF $\text{Ca}(\text{NO}_3)_2$	FLOWERS PRODUCING GYNOPHORES		FLOWER-TO-GYNOPHORE INTERVAL IN DAYS	
	GRAMS MOLS PER LITER	NUMBER*	ANTHESIS	AVERAGE	MODE
High nitrogen.....	0.01500	108	July 16-31	12.7	7
		156	Aug. 1-23	8.8	7
Medium nitrogen.....	0.00450	190	July 16-31	17.0	9
		314	Aug. 1-23	11.0	9
Low nitrogen.....	0.00225	64	July 16-31	16.4	9
		148	Aug. 1-23	10.9	9
Very low nitrogen.....	0.00113	61	July 16-31	11.3	9
	0.00032	None	Aug. 1-23	.....	.....

\* Totals for four plants from each series.

with distilled water, sterilized in an autoclave, and held at 28°C. during germination. Four days later, when the cotyledons were just emerging, six seedlings as nearly uniform as possible in appearance were transplanted to each jar containing sand prepared as indicated for experimentation. To stimulate vegetative growth, nutrient solution I (table 4) was applied daily for two weeks. On April 26, solution II was substituted and the concentration of its salts was doubled at the end of a week. Manganese, as  $\text{MnSO}_4$ , was applied at about 0.4 p.p.m. and boron, as  $\text{H}_3\text{BO}_3$ , was applied at about 0.3 p.p.m., each time nutrient solutions were given. Iron, as  $\text{FeCl}_3$ , was applied once or twice weekly at the rate of 1-2 p.p.m. for series showing



little vegetative growth and at 5 p.p.m. for those showing extensive growth.

Because of the early onset of blossoming, specific cultural treatments were established when the plants were a little less than four weeks old. At that time the plants were healthy and vigorously

TABLE 3  
SUMMARY OF CHEMICAL ANALYSES OF HIGH AND  
VERY LOW NITROGEN SERIES (1933)\*

SERIES	FI-BROUS ROOTS	PRI-MARY ROOTS AND HYPO-COT- YLS	STEMS	STEM TIPS	PETI- OLES	LEAF- LETS	FLOW- ER- BUD COM- POSITE	GYNO-PHORES		FRUITS		
								NON-FRUIT- ING	FRUIT BEAR- ING	SMALL	LARGE	
											SHELLS	SEEDS
High N..... Very low N..	Total sugars											
	2.16 8.76	4.09 7.62	5.13 4.95	1.53 2.17	1.53 1.25	1.12 1.59	2.34 2.27	1.65 1.83	3.79 3.47	11.16 8.92	10.73 11.66	2.87 5.04
	Starch and dextrins											
	0.37 1.99	7.01 11.86	7.77 7.89	2.81 7.97	1.64 10.66	6.01 26.23	5.03 6.16	9.00 5.98	17.60 8.14	10.03 17.06	2.94 2.83	8.52 15.32
	Total sugars, starch, and dextrins											
	2.53 10.75	11.10 19.48	12.90 12.84	4.34 10.14	3.17 11.91	7.13 27.82	7.37 8.43	10.65 7.81	21.39 11.61	21.19 25.98	13.67 14.49	11.39 20.36
	Ether extract											
	..... .....	..... .....	..... .....	..... .....	..... .....	..... .....	..... .....	1.33 1.45	0.73 0.85	1.40 2.45	0.59 trace	44.06 47.02
	Total nitrogen plus nitrates											
	1.82 1.52	1.20 0.70	1.07 0.64	4.51 1.47	1.57 1.01	3.38 1.74	2.70 1.70	3.11 2.30	1.97 1.39	5.36 3.96	1.89 0.93	4.45 2.96

\* All figures are for percentages of dry weight. Analytical methods were the same as for 1935 except that total nitrogen plus nitrates were determined by the salicylic acid method.

vegetative, each having seven to nine fully expanded leaves. To avoid the complication of fruit setting before the plants in series A, B, D, and E had reached the desired level of nutrition, flower buds or freshly opened flowers were removed for a time. Daily counts were taken of all flower buds or flowers produced whether or not these

were removed or allowed to remain on the plants. Gynophores were counted periodically in the preceding four series.

SERIES A: VERY HIGH NITROGEN, NON-FRUITFUL.—Each jar of this series was given nutrient solution A, continuously supplied (21) from one siphon. The solution was collected in 18-liter carboys as it drained from the jars, the pH of 7.1 restored with dilute  $\text{NH}_4\text{OH}$ , and the solution used again. Every seven to nine days the jars were thoroughly flushed with tap water, fresh nutrient solution allowed

TABLE 4  
NUTRIENT SOLUTIONS

NUTRIENT SOLUTION	CONCENTRATION OF SALTS (GM. MOLS PER LITER)							PH AT APPLI- CATION
	$(\text{NH}_4)_2\text{SO}_4$	$\text{NH}_4\text{NO}_3$	$\text{CaCl}_2$	$\text{Ca}(\text{NO}_3)_2$	$\text{KH}_2\text{PO}_4$	$\text{KCl}$	$\text{MgSO}_4$	
I.....	0.00010	.....	0.00090	.....	0.000225	0.000225	0.00045	6.9
II.....	0.00040	.....	0.00040	0.00040	0.00045	0.00060	0.00060	.....
A.....	0.0060	.....	0.00225	.....	0.001125	0.0030	0.0030	7.1
AA.....	0.0090	.....	0.00225	.....	0.001125	0.0030	0.0030	7.3
B.....	.....	.....	.....	0.0090	0.001125	0.0030	0.0045	4.5
C-I.....	.....	.....	.....	0.0045	0.001125	0.0030	0.0045	4.0
C-II.....	.....	0.00079	0.00079	0.00315	0.001125	0.0030	0.0030	5.3
DE.....	.....	.....	0.00180	.....	0.00090	0.00180	0.00180	4.3
High N, 1933.....	.....	.....	.....	0.01500	0.002250	0.002250	0.00450	4.1
Very low N, 1933*.....	.....	.....	.....	0.00032	0.000080	0.000080	0.00016	4.1

\* Formula for the nutrient solution used beginning August 1.

to drain through the sand, and the supply carboys filled with fresh nutrient solution for the next period. During the seven- to nine-day periods of repeated use of the nutrient solution, about 3 per cent of the ammonium nitrogen was daily oxidized to nitrate nitrogen.

The plants were given a six-hour day, from 8:30 A.M. to 2:30 P.M. Light intensity was reduced by muslin stretched 4 feet above the plants. During heavily clouded weather the canopy was rolled up. On very bright days it was supplemented with one or two layers of longcloth.

SERIES AA: VERY HIGH NITROGEN, LIMITED FRUITFULNESS.—Only two jars of plants were included in this test. Nutrient solution AA was supplied by the continuous application method used for

series A. Originally the former series was intended as a guide to determine whether the concentration of 0.0060 for  $(\text{NH}_4)_2\text{SO}_4$  planned for series A would be toxic. As the plants remained healthy the test was continued.

The plants were shaded by muslin about 1 foot above the rim of the jars and were exposed to the seasonal length of day. Muslin protected the plants from direct sunlight during the early morning and late afternoon.

**SERIES B: HIGH NITROGEN, LIMITED FRUITFULNESS.**—This series received nutrient solution B. The plants were moved out of the

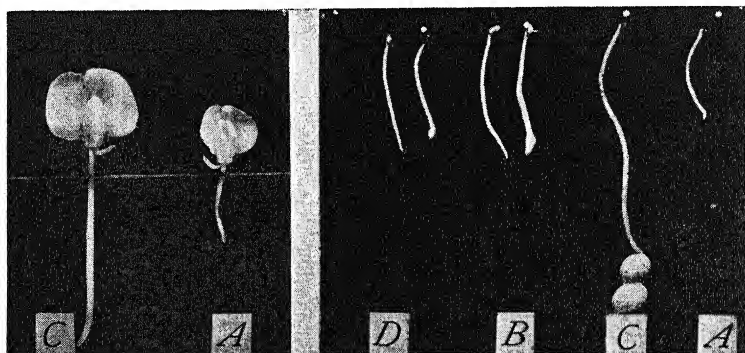


FIG. 1.—Flowers and gynophores produced in the several cultures: C, abundantly fruitful; A, very high nitrogen, non-fruitful; D, high carbohydrate; B, high nitrogen, limited fruitfulness.

dark house at 8:30 A.M. and back into it at 4:30 P.M., thus giving them an eight-hour day. Light intensities were greater than for series A, and were varied in a similar manner.

In this series the size of the flowers was used as an index to the nutritional level of the plants. It had been noticed that plants with a very limited accumulation of carbohydrates produced flowers about half as large as those on plants of only slightly greater carbohydrate reserve (fig. 1). Accordingly the nutrient solution and intensity of light were varied to maintain, as far as possible, the production of flowers of reduced size.

**SERIES C: ABUNDANTLY FRUITFUL.**—When the plants of this series were one month old, they were thinned to four per jar. Nutrient solution C-I was applied during the succeeding two and one-half

weeks, and solution C-II during the remainder of the experimental period. The concentration and quantity of nutrient solution were varied as necessary to maintain abundant production of flowers and gynophores. The plants were given full exposure to daylight.

**SERIES D AND E: HIGH CARBOHYDRATE.**—Both series were given the same treatment, except for slight variations noted hereafter. At the start of the treatments all nitrogenous salts were flushed from the sand with distilled water and nutrient solution DE. The concentrations of salts in this solution were very low, as ordinary concentrations had been found to be injurious and it was necessary to apply the solutions three times per week to stabilize the pH. Previous observations on high carbohydrate plants had shown that they soon accumulate ions to the point of toxicity, one of the most serious effects being the failure of such plants to accumulate any considerable amount of starch. When visible growth of the plants in these two series had practically ceased (June 9), the concentrations of all salts except  $\text{KH}_2\text{PO}_4$  were reduced to one-half that recorded for them in table 4 in order to minimize possible injurious effects.

No nitrogen was added at any time to the nutrient solution of series E. Small amounts of nitrate were added to series D when it seemed that flower production was about to cease. Between June 22 and July 19, 8 liters of nutrient solution DE with  $\text{Ca}(\text{NO}_3)_2$  at a concentration of 0.0001125 and 3 liters with this salt at a concentration of 0.000225 were added to each jar. Both series were exposed to the greatest available amount of direct sunlight.

In these series, as in series B, the size of the flowers was used as a criterion of nutritional level. About the time the lower leaves began to turn bright yellow and drop, the flowers became periodically much reduced in size and paler in color. Exfloration was discontinued in series D during the second cycle of floral reduction, and in series E during the third.

**CONTROL OF PH.**—On an average of once every two weeks, the pH of each jar in series B, C, D, and E was taken. At such times nutrient solution was applied to the cultures and the first 50 to 100 cc. of drip collected. All determinations were made by use of indicators on 2–3 cc. samples.

After determinations had been made, the pH was adjusted if nec-

essary with 0.001 N KOH or 0.006 N  $\text{H}_2\text{SO}_4$ . One or 2 liters of base or acid were added, followed by sufficient distilled water and nutrient solution to flush the sand. Table 5 summarizes the pH determinations.

It must be emphasized that each series was grown to secure a definite type response. Cultural treatment could not be a matter of inflexible routine therefore, for light, temperature, and relative humidity, which depend largely upon weather conditions, varied over a rather wide range. Accordingly it was necessary to modify cultural practice in order to produce the type responses desired.

TABLE 5  
SUMMARY OF AVERAGE PH DETERMINATIONS

PH OF NUTRIENT SOLUTION	SERIES					
	CONTINUOUS APPLICATION		PERIODIC APPLICATION			
	A	AA	B	C	D	E
Applied.....	7.1	7.3	4.0	5.2	4.3	4.3
Drip.....	5.5	5.5	.....	.....	.....	.....
First drip before flushing.....	.....	.....	5.0	5.7	4.8	5.1
Mean deviation of jars.....	.....	.....	$\pm 0.43$	$\pm 0.28$	$\pm 0.60$	$\pm 0.34$

COMPARATIVE CULTURES OF TOMATO.—For comparison a few tomato plants were included with each of the preceding treatments except AA. Two plants of the Marglobe variety were grown per jar, two jars having been included with series A and E and one with series B, C, and D. The number of plants used was limited.

#### CHEMICAL METHODS

HARVEST AND PRESERVATION.—An abundance of sunshine favored the accumulation of carbohydrates toward the close of the experiment. Records of the weather bureau at the University of Chicago show that, for the eleven days preceding the first day of harvest, there was a daily average of 11.8 hours of sunshine.

Harvesting was done from July 19 to 21. In all cases the plants were placed in the dark house overnight. The following morning

each series was removed just before dissection, carefully flushed from the jars, and sand adhering to the roots and fruits dislodged with a jet of tap water.

Dissections were made at once in the laboratory. All series were separated into root, stem, petiole, leaflet, and composite fractions. Hypocotyls were included with roots. The stem fraction consisted of all stem tissue save that of the youngest 2 cm., the stem tips. Only healthy leaflets were used in the leaflet fraction, the remainder of such healthy leaves comprising the petiolar fraction. The composite fraction consisted of all leaves that were discolored in any way, stem tips, axillary buds, and (in the cases of all series except B and C) the gynophores. The fruits of series B were too small and few to constitute a separate fraction, and hence were combined with the gynophores to make a single fraction. The gynophore fraction of series C contained not only gynophores which had not reached the sand, but also those with very slightly enlarged ovaries. The fruit fraction of series C included both the fruits proper and the gynophores on which they had formed. Only the stems of the tomato plants were harvested.

The fractions were minced, preserved in 80 per cent alcohol by standard procedure, and stored in the dark. About two hours elapsed from the time series C was flushed from the jars until the fractions were put into alcohol. The period of harvest for the other series averaged about one hour. Extractions were made during the two succeeding weeks by standard procedure (2), except that Schleicher and Schüll filter paper no. 595 was used instead of hardened filter paper, to facilitate the rate of filtration. Filtrates were brought to volume and aliquoted at 25°C. All filtrates were free from cloudiness except that of the fruits in series C, which had a fine and somewhat stable suspension that appeared oily in nature. The ether extract of this filtrate appears in table 8. The residues of alcoholic extraction were ground first in a drug mill and then for twelve hours in a ball mill, dried in a vacuum oven at 80°, and stored in desiccators.

CARBOHYDRATES.—Copper precipitations were made by the QUISUMBING-THOMAS method (16) and the copper determined by the volumetric permanganate method of the A.O.A.C. (1).

SUGARS.—Sugar determinations were made on 500 cc. aliquots of filtrates prepared for analysis as described by STUART (20). Analyses were made for total sugars on 110 cc. of the cleared filtrates. The solutions were made just acid to methyl red with 10 per cent acetic acid. Two drops of Difco invertase solution were added and the reducing power was determined after three hours of enzymatic action at 37°. Total sugars are expressed as invert sugars. Reducing sugars were determined only on the stems.

STARCH AND DEXTRINS.—Beakers of 250 cc. capacity covered with sections of petri dishes were used for gelatinization and digestion. Samples of the dried residues were taken up with about 20 cc. of distilled water and gelatinized by heating on a steam bath and for 45 minutes in an 80° oven. After cooling, 4 cc. of fresh saliva was added and the samples digested for 45 minutes at 37°. The digested samples were heated over a steam bath, filtered hot, and washed free from sugars. Sufficient HCl was added to the filtrates to produce an acid concentration of 2.5 per cent, and the samples refluxed in boiling water for two and one-half hours. After cooling, they were made alkaline to methyl red but just acid to litmus paper. Results are expressed as starch, using 0.93 as the factor.

“HEMICELLULOSES.”—The residues from which starch and dextrans had been removed were taken up in 2.5 per cent HCl, refluxed for two and one-half hours in boiling water, filtered, and cleared before analyses. Results are expressed as dextrose.

SOLUBLE NITROGEN.—Analyses were made on 100 cc. portions of the alcoholic extract by the reduced iron method (15).

INORGANIC NITROGEN.—The methods of STUART (20) and SESSIONS and SHIVE (18) were modified for the determination of ammonium and nitrate nitrogen. STUART's method of preparation was used on 200 cc. aliquots of the alcoholic extract reduced to 50 cc. of cleared extract.

Ammonium nitrogen was determined as described by STUART, except that an aeration period of one and one-half hours was allowed. In addition, the lower ends of the foam traps of test-tubes containing the samples were closed with relatively loose cotton plugs to trap the fine alkaline spray formed during rapid aeration. Determinations checked much more closely when cotton plugs were used

than when they were omitted. This was especially true for the determinations of nitrate nitrogen.

For nitrate nitrogen, the method of SESSIONS and SHIVE was adapted to the Van Slyke-Cullen urea apparatus. Analyses were made on the ammonium-free aliquots. Two cc. of 10 per cent NaOH and about 0.3 gm. of deVarda's alloy were added and aeration carried on at a fairly rapid rate for six hours. In preliminary tests nitrate nitrogen had been quantitatively recovered by this method.

**SOLUBLE ORGANIC NITROGEN.**—This fraction was calculated as the difference between soluble nitrogen and inorganic nitrogen.

**RESIDUAL NITROGEN.** The micro-Kjeldahl method outlined by PREGL (14) was used for nitrogen analyses of the alcohol-extracted residues.

## Results

### VEGETATIVE AND REPRODUCTIVE RESPONSES

Comparative vegetative and reproductive responses of the several series are illustrated in figures 1 and 2. The plants within each series presented a uniform appearance when harvested.

**SERIES A: VERY HIGH NITROGEN, NON-FRUITFUL.**—The plants in this treatment were dark blue-green in color and grew slowly. The stems were slender and weak, producing few branches. During hot days the leaflets wilted considerably even though completely protected from direct sunlight. Many of the older leaves gradually died from too restricted carbohydrate synthesis. The accumulation of carbohydrates was so limited that eleven of the plants lost practically all their leaves and were removed from the experiment. A large proportion of the leaves on the remaining plants were partially discolored or dead at harvest, thus producing a rather large composite fraction. Root systems, observed at harvest, were markedly limited in extent and lacking in mechanical strength. Despite the extreme nutritional level at which this series was maintained, an average of about one small flower per plant was produced during the course of the experiment. During the period from June 17 to July 18 no flowers were removed. Of the eighteen flowers allowed to remain on the plants, only one gave rise to a gynophore.

**SERIES AA: VERY HIGH NITROGEN, LIMITED FRUITFULNESS.**—This series resembled series B in general appearance, but branched



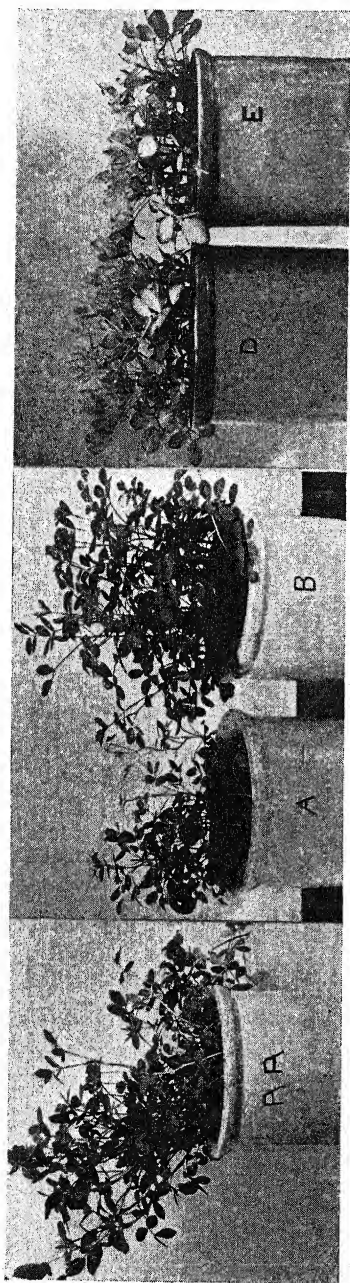


FIG. 2.—Plants in the several cultures employed: *AA*, very high nitrogen, limited fruitfulness; *D*, *E*, high carbohydrate, *B*, high nitrogen, non-fruitful.

more freely. In reproductive responses the plants were intermediate between series A and B, producing an average of 9.6 flowers and 2.3 gynophores per plant.

**SERIES B: HIGH NITROGEN, LIMITED FRUITFULNESS.**—Plants of this series were much more vegetative than those of series A. From June 4, when exfloration was discontinued, they produced an average of 16.5 flowers, 7.6 gynophores, and 0.5 small fruits.

**SERIES C: ABUNDANTLY FRUITFUL.**—Both vegetative and reproductive responses were greatest in this series. The plants were larger, thicker stemmed than those in all other series, much branched, and almost as blue-green in color as those of the preceding series. They produced an average of 116.8 flowers, 19.6 gynophores, and 28.4 fruits.

**SERIES D AND E.**—Within two weeks after series D and E were flushed free from available nitrogen they had become conspicuously light green. A few days later the oldest leaflets became pale along their midribs and larger veins, remaining greenest at their margins. Shortly thereafter those slightly younger underwent

the same color change and the oldest ones turned bright yellow and dropped. Leaves formed after this time were few in number and uniformly pale green in color. In both series the diameters of the

TABLE 6  
PERCENTAGE DRY MATTER

PLANT FRACTION	SERIES					
	A	AA	B	C	D	E
Roots.....	7.22	9.02	8.37	9.25	.....*	9.86
Stems.....	13.70	13.87	16.19	21.81	25.16	26.90
Petioles.....	10.23	10.56	12.37	17.20	17.92	18.58
Leaflets.....	13.33	14.04	15.53	17.38	18.28	18.71
Green gynophores..				10.53		
Fruits.....				31.60		
Gynophores and fruits.....			16.30			
Composite.....	13.44	12.32	16.54	15.04	21.22	20.63
Tomato stems.....	6.17	.....	.....*	14.98	24.24	19.46

\* Soluble solids were lost.

TABLE 7  
PERCENTAGE SOLUBLE SOLIDS IN TOTAL SOLIDS

PLANT FRACTION	SERIES					
	A	AA	B	C	D	E
Roots.....	18.85	18.42	19.94	22.43	.....*	27.19
Stems.....	18.20	17.73	19.23	19.52	14.56	13.95
Petioles.....	31.19	30.07	29.36	25.99	21.20	20.16
Leaflets.....	30.89	27.52	27.56	27.78	26.21	26.62
Green gynophores..				27.37		
Fruits.....				14.42		
Gynophores and fruits.....			29.23			
Composite.....	30.48	28.61	27.75	31.41	23.33	23.58
Tomato stems.....	39.68	.....	.....*	29.71	19.80	27.54

\* Soluble solids were lost.

stems were intermediate between those of plants in series A and C. In general appearance and texture the plants were typically high carbohydrate.

TABLE 8  
CARBOHYDRATE AND ETHER EXTRACT FRACTIONS  
(PERCENTAGE DRY WEIGHT)

PLANT FRACTION	CHEMICAL FRACTIONS	SERIES					
		A	AA	B	C	D	E
Roots	Total sugars.....	0.06	0.19	2.12	6.50	.....	14.43
	Starch and dextrins...	2.94	2.56	2.68	1.96	(9.85)*	8.37
	Sub-totals.....	3.00	2.75	4.80	8.46	.....	22.80
	Hemicelluloses.....	13.15	13.46	12.81	11.20	(16.84)	11.40
	Totals.....	16.15	16.21	17.61	19.66	.....	34.20
Stems	Reducing sugars.....	None	None	0.20	2.34	1.82	1.35
	Total sugars.....	0.96	1.40	3.33	7.77	5.39	4.85
	Starch and dextrins...	1.53	1.85	3.03	2.09	21.42	28.08
	Sub-totals.....	2.49	3.25	6.36	9.86	26.81	32.93
	Hemicelluloses.....	11.96	12.29	12.35	14.19	11.64	10.44
Petioles	Totals.....	14.45	15.54	18.71	24.05	38.45	43.37
	Total sugars.....	0.28	0.65	1.25	3.11	1.28	1.43
	Starch and dextrins...	3.71	4.01	3.44	2.85	7.06	8.35
	Sub-totals.....	3.99	5.26	4.69	5.96	8.34	9.78
	Hemicelluloses.....	8.49	9.04	8.77	10.61	10.52	11.18
Leaflets	Totals.....	12.48	14.30	13.46	16.57	18.86	20.96
	Total sugars.....	0.02	0.17	0.34	1.15	0.85	1.40
	Starch and dextrins...	1.14	1.51	1.93	4.40	13.42	10.66
	Sub-totals.....	1.16	1.68	2.27	5.55	14.27	12.06
	Hemicelluloses.....	4.28	4.38	4.19	5.20	6.41	5.97
Gynophores and fruits	Totals.....	5.44	6.06	6.46	10.75	20.68	18.03
	Total sugars.....	.....	.....	5.55	.....	.....	.....
	Starch and dextrins...	.....	.....	8.88	.....	.....	.....
	Sub-total.....	.....	.....	14.43	.....	.....	.....
	Hemicelluloses.....	.....	.....	7.03	.....	.....	.....
Green gynophores	Total.....	.....	.....	21.46	.....	.....	.....
	Ether extract.....	.....	.....	6.28	.....	.....	.....
	Total sugars.....	.....	.....	.....	2.82	.....	.....
	Starch and dextrins...	.....	.....	.....	6.04	.....	.....
	Sub-total.....	.....	.....	.....	8.86	.....	.....
Fruits	Hemicelluloses.....	.....	.....	.....	9.42	.....	.....
	Total.....	.....	.....	.....	18.28	.....	.....
	Ether extract.....	.....	.....	.....	1.63	.....	.....
	Total sugars.....	.....	.....	.....	6.47	.....	.....
	Starch and dextrins...	.....	.....	.....	6.89	.....	.....
Fruits	Sub-total.....	.....	.....	.....	13.36	.....	.....
	Hemicelluloses.....	.....	.....	.....	4.69	.....	.....
	Total.....	.....	.....	.....	18.05	.....	.....
	Ether extract.....	.....	.....	.....	38.56†	.....	.....

\* Percentages in parentheses are calculated on basis of residues of alcoholic extraction.

† Alcoholic soluble extract, 2.95%; alcoholic insoluble extract, 35.61%.

TABLE 8—*Continued*

PLANT FRACTION	CHEMICAL FRACTIONS	SERIES					
		A	AA	B	C	D	E
Composite	Total sugars.....	0.02	0.75	1.10	2.21	2.05	2.14
	Starch and dextrins...	1.85	2.74	2.85	5.90	13.72	14.45
	Sub-totals.....	1.87	3.49	3.95	8.11	15.77	16.59
	Hemicelluloses.....	5.70	5.85	6.44	7.56	7.48	5.54
	Totals.....	7.57	9.34	10.39	15.67	23.25	22.13
Tomato stems	Total sugars.....	0.85	.....	.....	20.46	12.34	17.70
	Starch and dextrins...	3.15	.....	(3.22)	9.24	15.76	21.55
	Sub-totals.....	4.00	.....	.....	29.70	28.10	39.25
	Hemicelluloses.....	8.68	.....	(15.83)	11.75	13.18	11.94
	Totals.....	12.68	.....	.....	41.45	41.28	51.19

SERIES D: HIGH CARBOHYDRATE, GYNOPHORE-PRODUCING.—These plants were identical in appearance with those of series E until about eight days after the first addition of  $\text{Ca}(\text{NO}_3)_2$  to their nutrient solution. During the remainder of the experiment, the first three weeks of July, a few new leaves appeared and the plants as a whole became slightly greener. No nodules were visible at harvest on any of the high carbohydrate plants, except for a few small ones near the hypocotyl of a single plant.

For the period during which flowers were no longer removed until the application of  $\text{Ca}(\text{NO}_3)_2$  caused an increase in blooming (June 6 to July 1), the plants produced an average of 6.8 flowers; during the remainder of growth (July 2–18) the average was 10.3 flowers. Only one gynophore had been produced by June 18, a total of twenty by July 4, and of twenty-three by July 20 by the forty-eight plants in this series. Enlargement of ovaries was scarcely perceptible.

SERIES E: VERY HIGH CARBOHYDRATE, NON-FRUITFUL.—From July 5 to 18 no blossoms were removed from the plants of this series. An average of 4.1 flowers was produced per plant. Only one plant in the entire lot developed a gynophore.

COMPARATIVE CULTURES OF TOMATO.—Although the vegetative responses of tomatoes were similar to those of peanuts, reproductive behavior was quite different. Tomato plants of series A were moderately vegetative, medium green in color, and very succulent. During the period of much reduced illumination in the earlier part of

TABLE 9  
NITROGEN FRACTIONS (PERCENTAGE DRY WEIGHT)

PLANT FRACTION	CHEMICAL FRACTIONS	SERIES					
		A	AA	B	C	D	E
Roots	Ammonium N.....	0.113	0.276	0.035	0.025	.....	None
	Nitrate N.....	0.130	0.190	0.310	0.040	.....	None
	Soluble organic N.....	0.673	0.395	0.218	0.146	.....	0.007
	Residual N.....	1.913	1.880	1.571	1.638	(1.003)	0.886
	Total N.....	2.829	2.741	2.134	1.849	.....	0.893
Stems	Ammonium N.....	0.067	0.049	0.025	0.009	None	None
	Nitrate N.....	0.470	0.610	1.010	0.060	None	None
	Soluble organic N.....	1.086	0.568	0.233	0.087	0.069	0.065
	Residual N.....	1.143	1.102	1.022	0.645	0.357	0.326
	Total N.....	2.766	2.329	2.290	0.801	0.426	0.391
Petioles	Ammonium N.....	0.096	0.048	0.042	0.027	None	None
	Nitrate N.....	0.990	1.120	1.060	0.110	None	None
	Soluble organic N.....	1.240	0.490	0.817	0.096	0.117	0.094
	Residual N.....	1.845	1.599	1.606	0.874	0.602	0.555
	Total N.....	4.171	3.257	3.525	1.107	0.719	0.649
Leaflets	Ammonium N.....	0.100	0.049	0.046	.....	None	None
	Nitrate N.....	0.280	0.310	0.460	0.367	None	None
	Soluble organic N.....	1.320	0.563	0.629	.....	0.195	0.179
	Residual N.....	4.717	4.447	4.426	3.130	1.114	1.205
	Total N.....	6.417	5.369	5.561	3.497	1.309	1.384
Gynophores and fruits	Ammonium N.....	.....	.....	0.010	.....	.....	.....
	Nitrate N.....	.....	.....	0.390	.....	.....	.....
	Soluble organic N.....	.....	.....	1.188	.....	.....	.....
	Residual N.....	.....	.....	2.839	.....	.....	.....
	Total N.....	.....	.....	4.411	.....	.....	.....
Green gyno- phores	Ammonium N.....	.....	.....	.....	0.030	.....	.....
	Nitrate N.....	.....	.....	.....	0.110	.....	.....
	Soluble organic N.....	.....	.....	.....	1.143	.....	.....
	Residual N.....	.....	.....	.....	1.721	.....	.....
	Total N.....	.....	.....	.....	3.004	.....	.....
Fruits	Ammonium N.....	.....	.....	.....	0.015	.....	.....
	Nitrate N.....	.....	.....	.....	0.020	.....	.....
	Soluble organic N.....	.....	.....	.....	0.160	.....	.....
	Residual N.....	.....	.....	.....	2.327	.....	.....
	Total N.....	.....	.....	.....	2.522	.....	.....
Composite	Ammonium N.....	0.174	0.073	0.055	0.044	None	None
	Nitrate N.....	0.290	0.280	0.720	0.100	None	None
	Soluble organic N.....	1.470	0.763	0.517	0.367	0.257	0.221
	Residual N.....	3.162	3.033	2.760	2.912	1.127	1.034
	Total N.....	5.096	4.149	4.824	3.423	1.384	1.255

TABLE 9—*Continued*

PLANT FRACTION	CHEMICAL FRACTIONS	SERIES					
		A	AA	B	C	D	E
Tomato stems	Ammonium N. ....	1.259	.....	.....	0.262	None	None
	Nitrate N. ....	0.820	.....	.....	0.040	None	None
	Soluble organic N. ....	2.116	.....	.....	0.707	0.201	0.221
	Residual N. ....	1.163	.....	(1.258)	0.853	0.500	0.421
	Total N. ....	5.358	.....	.....	1.862	0.701	0.642

the experiment, they developed two to three flower buds per cluster, which remained very small and eventually dried and fell off. Later light intensity was slightly increased and the buds formed were larger and persistent. The series B plants were vigorously vegetative, dark green in color, succulent, and but sparingly branched. They produced clusters of large flowers which fell shortly after opening. Even though the plants of series C did not grow so tall as those of series B, they were profusely branched and bore a relative abundance of flowers and fruits. Series D and E were almost identical in general appearance, growing very little and becoming yellowish and woody. The flower buds of series D, however, were slightly larger than those of series E. One plant of series E blossomed, bore one small fruit and died.

#### CHEMICAL DATA

Data of percentage dry matter are given in table 6, of percentage soluble solids in total solids in table 7, of carbohydrate and ether extract analyses in table 8, and of nitrogen fractions in table 9.

#### Discussion

##### FLOWER-TO-GYNOPHORE INTERVAL

It is apparent that the enlargement of fruits noticeably increased the average flower-to-gynophore interval but did not change the mode of this interval.

In certain preliminary experiments it had been observed that the average time required for the appearance of the first gynophore on a plant was about one week after its flower had opened. The enlargement of fruits, however, was found to increase the average interval

approximately 50 per cent, as shown by the data for July 16 to 31 in table 1. The records in this table are for plants which had commenced to bloom about June 14, so that fruit enlargement was in progress when the first flowers were tagged for a study of the interval. The period between the opening of flowers and the appearance of gynophores became shorter again during August, because most of the gynophores produced during July and August arose from stems which drooped over the rims of the jars and consequently did not set fruits.

When the data are analyzed on the basis of statistical modes, there appears to be a much greater degree of stability for the period between anthesis and the initiation of meristematic activity in the gynophore than the data of averages indicate. Table 1 shows that (1) within each series the statistical mode of this period was not altered by the onset of fruit development, having maintained itself at seven days in the high nitrogen series and at nine days in each of the other three series throughout the period of observation; and (2) with the possible exception of the high nitrogen series, there was no correlation between the mode of the interval and the amount of nitrate supplied.

BEHAVIOR OF VERY LOW NITROGEN PLANTS.—From the preceding comments on modal stability, it is evident that, for plants producing gynophores, the general level of nutrition has but little influence on the rate of their appearance. If very low nitrogen plants bearing gynophores and fruits have their supply of available nitrogen sufficiently reduced, however, the nutritional level of such plants will fall below the threshold of meristematic activity in the gynophore initials. This change occurred in the very low nitrogen plants as recorded in table 1. Until August 1 they produced gynophores with a modal frequency equal to that of the medium nitrogen series; but after August 1 they developed no gynophores at all, although they continued to bloom sparingly.

The response of the plants in series D is also significant in this respect. They were below the threshold of gynophore production when the application of minute quantities of nitrate was started on June 22. By July 4 the forty-eight plants in the series had produced a total of twenty gynophores; but only three additional gynophores

had formed by July 20, although the intermittent application of nitrate was continued until that date. These responses indicate that the nutritional level of most of the plants was raised above the threshold of gynophore initiation for a period of not more than twelve days (from June 22 to July 4), and that the extension of gynophores soon caused the nutritional level to drop again below that threshold.

**PRECOCITY OF GYNOPHORE DEVELOPMENT.**—Correlation of the preceding observations indicates a definite precocity of gynophore development for plants representing a wide range of nutritional levels; that is, if gynophores are formed at all, the modal frequency of their flower-to-gynophore intervals will be confined to a very narrow range not exceeding a period of nine days.

Lack of sufficient data makes it impossible to extend the application of this concept to very high nitrogen plants such as those of series A and B.

#### RELATIVE REPRODUCTIVITY OF SERIES B AND C

The data given under "Vegetative and reproductive responses" for the fruiting behavior of series B are apt to be misleading, as they are based on the numbers of gynophores and fruits. Data comparing average weights of these plant parts with those of series C are significant. On a green weight basis the average weights of non-fruiting (green) gynophores in series B and C were 0.06 and 0.13 gm. respectively, and of fruits 0.4 and 1.02 gm. As each plant of series B produced an average of only 0.2 gm. of fruit and each one of series C an average of 29.02 gm., the former may be considered relatively non-fruitful.

#### STABILITY OF REPRODUCTIVE FUNCTION

In this discussion of stability emphasis is placed on the opening of flower buds and the initial stages of fruit formation. These two phases of reproduction are less readily modified in the peanut plant than in the tomato, and may be summarized as follows. In series A (very high nitrogen) the peanut bloomed sparingly and was non-fruitful, but the tomato failed even to open flower buds. In series B (high nitrogen) the peanut produced many flowers and a few small fruits, while the tomato blossomed and remained entirely non-fruitful. In series D (high carbohydrate) the peanut bore numerous



flowers and a few gynophores, but the tomato gave rise to flower buds only. In series E (very high carbohydrate) the peanut blossomed freely and was non-fruitful, while reproduction in the tomato was limited to the formation of a few small flower buds. These results show that the reproductive function is more readily suppressed in the tomato than in the peanut.

#### GENERAL TRENDS OF CHEMICAL DATA

Table 6 shows a consistent increase in the percentage dry matter from highest nitrogen to highest carbohydrate series for all plant fractions except the composites of series A and B. The latter are relatively high in percentage dry matter, largely because the leaflets included had a higher proportion of discolored and partially desiccated tissue.

The percentage of soluble solids in total solids tends to decrease from highest nitrogen to highest carbohydrate series, with the notable exception of the root fractions, which have an inverse correlation.

The following trends are evident in tables 8 and 9. From highest nitrogen to highest carbohydrate series soluble and residual nitrogen decrease and total carbohydrates increase. These trends agree with those of investigations on other species not conditioned by photoperiodic behavior or by specific temperature treatments.

There is no uniformity of opinion as regards the relative importance of the several carbohydrate and nitrogen fractions in their relations to vegetation and reproduction of plants (5, 8). Lack of concordance in viewpoints is in part associated with differences inherent in the plants which have been studied. Some of them are normally annual; many are herbaceous but potentially perennial; others are woody perennials which have alternate periods of dormancy and growth (13, 20), and which may be subject to cumulative effects at present undefined. In photoperiodic response they represent short day plants, indeterminate bloomers, and those which flower only under long day conditions (10). Many plants are characterized by asexual storage organs, the enlargement of which may modify the interpretation of chemical data. Certain plants are highly sensitive to range of temperature (11, 24, 25). Because of these and other in-

herent differences, it is to be anticipated that the vegetative and reproductive responses will not always be correlated with exactly the same chemical fractions.

Most of the factors specifically mentioned in the preceding discussion were not limiting in the present study of the peanut. It is neither an annual plant nor one sufficiently hardy to exhibit the growth rhythm of a woody perennial, but continues to fruit indefinitely under certain conditions. It has proved to be an indeterminate bloomer. None of its asexual parts become especially enlarged for food storage. When given an adequate supply of water and mineral salts, it grew luxuriantly under the conditions of temperature prevailing during the period of experimentation.

The significant chemical fractions in the peanut plant are considered to be (1) total sugars, starch, and dextrans taken as a unit; and (2) total nitrogen.

CARBOHYDRATE FRACTIONS.—In correlating chemical data in nutritional studies, such as the one reported here, it seems logical to include only those chemical fractions which correspond to the vegetative and reproductive responses obtained. On this basis total sugars, starch, and dextrans (table 8) considered together are the significant carbohydrates; for neither total sugars alone nor starch and dextrans alone are consistently correlated with the responses observed. Although "hemicelluloses" were determined on all plant fractions, their ranges in percentage from highest to lowest nitrogen series were much too small to regard this chemical fraction of any importance in the peanut.

NITROGEN FRACTIONS.—The choice of significant nitrogen fractions was not restricted to the preceding basis of correlation. Although the determinations for residual and soluble organic nitrogen (table 9) correspond, on the whole, to the nutritional levels of their respective cultural treatments, those of the inorganic forms do not. On the basis of correlation alone, only the assimilated nitrogen should be used for interpretive purposes. The inorganic fractions are included in the interpretation presented because they aid in maintaining metabolic processes at approximately a given level. A common case in point is that of a medium nitrogen plant growing under conditions favorable to carbohydrate synthesis. So long as such a

plant is given an adequate supply of inorganic nitrogen its nutritional level will undergo little change. But if the plant is suddenly deprived of all externally available nitrogen, it soon becomes high carbohydrate. A progressive change in its nutritional level occurs as the inorganic nitrogen in its tissues becomes metabolized without being replaced by absorption from the soil solution. Even though the inorganic nitrogen fractions in plant tissues may at times be present in excess of immediate needs for maintenance of a nutritional level, at least part of such nitrogen functions in this manner in intermediate bloomers.

The influence of inorganic nitrogen in plant tissues on the principal phases of carbohydrate metabolism is a relatively new field of investigation. HAMNER (3) has recently reported that nitrates have a direct influence on the rate of respiration in wheat. The effects of inorganic nitrogen on photosynthesis need careful study.

In view of the preceding contingencies, total nitrogen, rather than any specific nitrogen fraction, should be used in correlating nutritional studies of the peanut and of other plants of similar physiological constitution.

INTER-SPECIAL CORRELATION OF CHEMICAL DATA.—When the analyses for tomato and peanut stems from any specific cultural treatment are compared, two correlations appear. Data in table 9 show that, within each cultural treatment, the tomato maintained approximately twice the concentration of total nitrogen found in the peanut. On the other hand, no such consistent inter-special correlation exists in the data for carbohydrates (table 8). Accordingly when the two species are considered together, nutritional levels are more closely associated with the nitrogen than with the carbohydrate fraction.

#### CORRELATION OF VEGETATION AND REPRODUCTION

In the five main cultural treatments, vegetative activity increased from very high nitrogen to abundantly fruitful plants and decreased from the latter to very high carbohydrate plants. Gynophore and fruit production followed similar quantitative changes. The setting and enlargement of fruits did not obviously retard the rate of vegetative extension of the fruitful plants in series C. For the peanut,

then, vegetation and reproduction are not opposing tendencies, but are complements in the course of development. This is true also for the tomato (5).

#### MISCELLANEOUS OBSERVATIONS

A few grams of coarsely ground seed of the high and very low nitrogen series of 1933 have been kept in 250 cc. bottles since the time of harvest. In less than a year the high nitrogen sample had become rancid in odor and taste, but the very low nitrogen sample was still free from rancidity after a period of three years. The seed of the high nitrogen series (1933) had slightly less ether extract than that of the very low nitrogen series (table 3).

A distinct gradation of pigmentation in seed coats was observed throughout the four series grown in 1933. The seed coat color of the highest nitrogen series was the usual rich brown, that of the very low nitrogen culture was pale and almost pink in color. If the color of the seed coat is to be employed in the classification of varieties (19), it will be necessary to grow all the plants under the same environmental conditions.

As the plants in series D and E approached the high carbohydrate condition, the new leaflets were observed to have numerous marginal hairs or trichomes. At the time most of these plants were harvested, a jar of six plants was given an abundance of available nitrogen and placed in the shade. Leaf primordia formed after the resumption of growth produced smooth-margined leaflets only. The test was repeated on other high carbohydrate plants with the same results. The culture of six plants of series E was modified by giving them an excess of the chloride ion for a period of forty days. One result of this modification was the almost complete disappearance of starch from the stems. When these plants were abundantly supplied with nitrogen even the first leaflets to expand were free from marginal ciliation. Leaflets of high nitrogen plants (series A, AA, and B) were smooth-margined, and those of series C bore scattered hairs much less conspicuous than those of series D and E. These results indicate that the growth of marginal hairs is associated with a relatively high carbohydrate level of metabolism.

### Summary

1. In the peanut plant, greatest vegetative extension and fruitfulness occurred in the same cultural treatment.

2. All high nitrogen plants were dark blue-green in color, slender stemmed and succulent; all high carbohydrate plants were very pale yellow-green, relatively thick stemmed, and firm in texture. Both were weakly vegetative and non-fruitful.

3. From highest nitrogen to highest carbohydrate series the percentage dry matter and total carbohydrates increased, and the percentage soluble solids and total nitrogen decreased consistently with vegetative and reproductive responses. The root fractions increased in percentage soluble solids.

4. "Hemicelluloses" were not correlated with levels of nutrition.

5. The high carbohydrate condition favored a slightly greater percentage of ether extract in the seeds and gynophores, the suppression of rancidity in the seeds, and the development of marginal hairs on the leaflets.

6. Gynophore development was precocious. The mode of the flower-to-gynophore interval was only slightly influenced by the enlargement of fruits on older gynophores or by extensive variations in metabolic levels, but elongation of the gynophores was abruptly inhibited by extreme levels of nutrition.

7. The fruiting tendency was less sensitive to nutritional change in the peanut than in the tomato.

8. The concentration of the phosphate ion in nutrient solutions must be greatly reduced to obviate injury when the peanut plant is grown in the light.

9. Ammonium nitrogen nutrient solutions which do not precipitate when applied at pH 7.1 are reported.

10. A method for determining nitrates by a six-hour aeration period is described.

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# HISTOLOGICAL AND MICROCHEMICAL STUDIES OF THE REACTIONS OF TOMATO PLANTS TO INDOLEACETIC ACID

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(WITH TWENTY FIGURES)

## Introduction

Histological studies of bean plants which had been treated with indoleacetic acid were recently made at the United States Horticultural Field Station at Beltsville, Maryland, and at the University of Chicago (1). These results made it desirable that similar work be done with other plants that respond to indoleacetic acid. The tomato, which has been used extensively as a test plant in physiological studies on the effects of growth promoting substances, was chosen as a second subject for histological study. A comparison of the histological responses of tomato with those of bean is particularly desirable because of the fundamental difference in arrangement of tissues in the two plants, the tomato stem having an internal phloem while the bean stem does not.

**MATERIAL AND METHODS.**—Seedlings of the Pritchard variety were grown during the fall and winter months in the greenhouse at the United States Horticultural Field Station. They were started in flats and transplanted to small pots, from which they were successively shifted to larger ones as their size demanded. They were subjected to the natural light intensity and day length prevailing in the latitude of Beltsville. Environmental conditions were such that good growth was maintained.

A mixture of 20 mg. of indoleacetic acid in 1 gm. of lanolin was applied to the cut surface of stems decapitated in the mid portion of the internode above the second lobed leaf when the plants were expanding their fourth lobed leaf. Differentiation of the primary tissues of the internode treated was definite, but little or no secondary

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activity was apparent. Additional plants were decapitated and kept as controls, pure lanolin being smeared over the cut surface of some, and the rest being left with the cut surfaces untreated.

Specimens were collected at intervals of 6 hours for the first 72 hours and every 12 hours thereafter until 216 hours had elapsed. Material for histological purposes was fixed in Navashin's solution and imbedded by the butyl alcohol-paraffin method. Sections were cut at  $10\ \mu$  and stained with the triple stain. Comparable samples were examined microchemically immediately after each collection.

### Gross responses to treatment

The first change to be observed after application of the indoleacetic acid mixture to the stem is usually an epinastic movement of one or more leaves next below the point of application. This frequently takes place within a few hours after treatment. There is usually no bending of the stem but there is a loss of color for a distance of 3 to 5 mm. below the cut surface. At the end of about two days the upper part of the stem shows evidence of enlargement and further loss of color. By the end of a week small bumps at the periphery of the swollen region mark the positions of adventitious roots, which emerge under favorable conditions. The portion of the internode below the swollen area retains its natural color and continues to grow, for a time at least, at approximately the same rate as the internode just below it. This same region of the controls, however, whether treated with pure lanolin or left untreated, fails to continue to enlarge and becomes woody.

The indoleacetic acid does not inhibit the development of buds below the treated area. In the plants treated terminally and in their controls these buds appeared to start growth immediately, and in a short time the uppermost one or two of them assumed the lead and produced vigorous branches (fig. 1A).

### Histological observations

The epidermal cells covering the area of greatest activity show no striking response to indoleacetic acid other than a slight enlargement. They are stretched and sometimes ruptured by the expansion of tissues inclosed by them.

The cortex of an untreated stem consists of four regions: an outer layer of chlorophyll bearing cells, a zone of collenchyma, a layer of parenchyma, and an endodermis. The chlorophyll bearing cells are small and thin walled, with extensive intercellular spaces. In treated stems these cells begin to increase in size within 24 hours, and even-

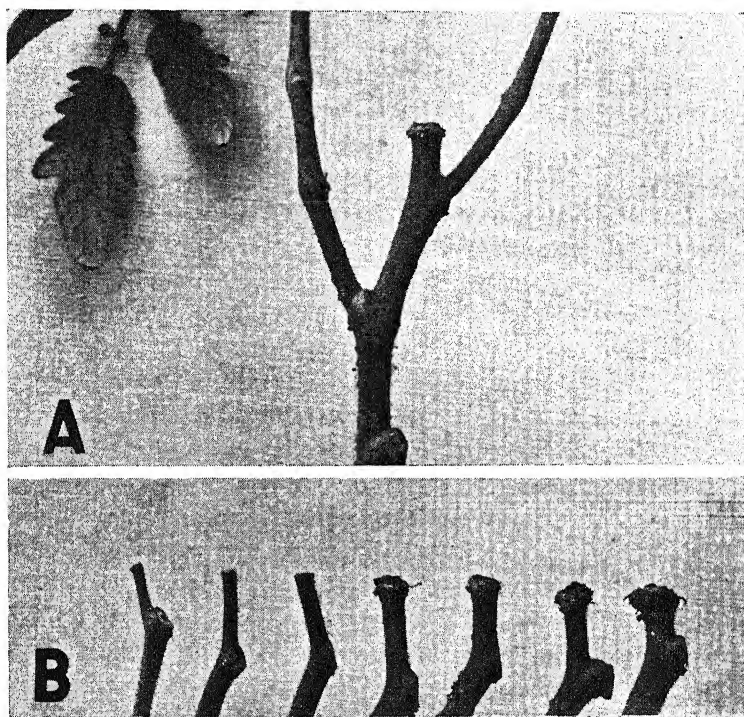


FIG. 1.—*A*, 10 days after decapitation of stem at center and treatment of same with indoleacetic acid-lanolin mixture. Strong lateral growths have developed from lower nodes. *B*, at left three stems decapitated and treated with lanolin only; at right four stems treated with indoleacetic acid mixture. All 10 days after treatment.

tually their radial diameter becomes two or three times that of those untreated. The walls remain thin and the intercellular spaces become less prominent. The chloroplasts become much less evident than in untreated material, which accounts for the paler green color of the treated areas.

The collenchyma of the cortex of untreated stems occurs as a

continuous layer two to five cells thick (fig. 2). Its cells are approximately isodiametric in cross section and their length is ten to fifteen times their diameter. Following treatment applied to a terminally cut surface, the collenchyma cells enlarge radially within the first 24 hours (fig. 3). At the end of about 54 hours, divisions in these cells in the upper 1 or 2 mm. of stem become abundant. These divisions are seen much more frequently in longitudinal than in cross sections, although they are by no means uncommon in the latter.

Centripetal to the collenchyma there are one to three layers of parenchyma. In treated stems these cells enlarge greatly, and about 54 hours after treatment they are in a very active state of division, the planes of division occurring in any direction (fig. 4).

Approximately 30 hours after treatment the cells of the endodermis begin to undergo tangential division, and by 54 hours divisions are very frequent and in all planes. The activity in the endodermis is much more pronounced than in the rest of the cortex, and this results in the formation of a band of highly meristematic tissue two to five cells wide. In the region where adventitious roots are formed, parts of this band of endodermal tissue are pushed out as coverings over the tips of the roots.

The cells of the pericycle which would normally mature as fiber cells fail to respond markedly to applications of the indoleacetic acid mixture, although after treatment they may enlarge somewhat and the walls of the potential fibers may thicken slightly. Many of the parenchymatous cells are apparently crushed by the activity of the phloem and endodermal tissues adjacent to them (figs. 10, 11). Some of those which were embryonic at the time the application was made do enlarge and undergo several divisions, but the derivatives of these play a relatively unimportant part in the development of the tumor or in the formation of adventitious roots.

The parenchyma of the external phloem exhibits a marked sensitivity to applications of the indoleacetic acid mixture. The tissues immediately adjacent to the treated surface show little change, even after a number of days (fig. 9A). Within 54 hours, however, the parenchymatous cells of the phloem lying within 2 mm. of the treated surface begin active divisions in all planes (fig. 4A). It is usually

from some of the derivatives of these cells that the external adventitious roots eventually become differentiated (fig. 11A, B). Other cells may differentiate as large tracheids, strands of sieve tubes and companion cells, or they may remain meristematic. In these experiments the activity in the external phloem did not extend more than 3 mm. from the treated surface, and the greatest activity occurs between 0.6 and 2.0 mm.

The internal phloem is as sensitive in its response to the treatment as is the external. There is little or no activity immediately adjacent to the cut surface, but within 54 hours those parenchymatous cells which lie about 1.0 mm. below the treated surface become very active (fig. 4); and later, as cell divisions continue, it is sometimes difficult to distinguish their derivatives from those of adjacent, actively dividing pith cells (fig. 8). Derivatives of these internal phloem parenchyma cells often differentiate as tracheids. Activity in the internal phloem extends farther down the stem as a result of the treatment than does the activity in any other tissue. Certain of the parenchymatous cells at levels as distant as 4 mm. from the treated surface undergo a few divisions and tracheids are derived from them.

The xylem parenchyma becomes relatively active as a result of treatment and this results in lateral displacement of many of the conducting elements. This activity is confined to portions of the stem 0.25 to 2.5 mm. from the treated surface.

Within 54 hours after treatment, the cells of the pith adjacent to the protoxylem points show considerable activity, and shortly thereafter many dividing cells are apparent throughout the pith region at distances of 0.5 to 2.0 mm. from the treated surface. The pith as a whole does not become meristematic beyond these limits, and most of the cells in the meristematic region undergo only a few divisions.

At the time of the treatment, certain of the pith cells adjacent to the sieve tubes and companion cells of the internal phloem had started differentiation as internal fibers. These cells do not become meristematic as a result of the treatment, and continue at a slow rate their differentiation as fibers.

The cells of the pith and rays adjacent to the primary xylem become relatively more active, and the activity extends farther from

the cut surface than is true for the pith as a whole. Those cells lying centripetally to the internal phloem become very active (figs. 8*B*, 9*B*); and it is from these derivatives, together with the derivatives of the internal phloem region, that the internal adventitious roots are differentiated.

### Microchemical observations

#### METHODS

**NITRATES.**—Diphenylamine reagent (0.1 gm. diphenylamine in 10 ml. of 75 per cent  $\text{H}_2\text{SO}_4$ ) was applied to fresh sections. Observations were made immediately and the intensity of the blue color that developed in various sections was made a basis of comparison.

**PROTEIN.**—Millon's reagent was applied to the sections. Immediately after slight heating, observation of the red precipitate was made.

**STARCH.**—Sections were treated with iodine potassium iodide (0.3 gm. I, 1.5 gm. KI, diluted to 100 ml.).

#### RESULTS

**NITRATES.**—The concentration of nitrates diminished during the experiment in both the treated and the untreated stems. However, there was more nitrate in the control. As the stem treated with the indoleacetic acid mixture began to swell, the nitrates disappeared entirely from the swollen area but were often abundant in the tissues just below.

**PROTEIN.**—The protein content of the treated stem increased in those areas where cell divisions became most abundant, while the protein content of the control stem remained rather constant during the experiment. Tissues of the stem in which marked meristematic activity subsequently occurred could readily be localized by an increase in the protein concentration, even before any cell divisions took place.

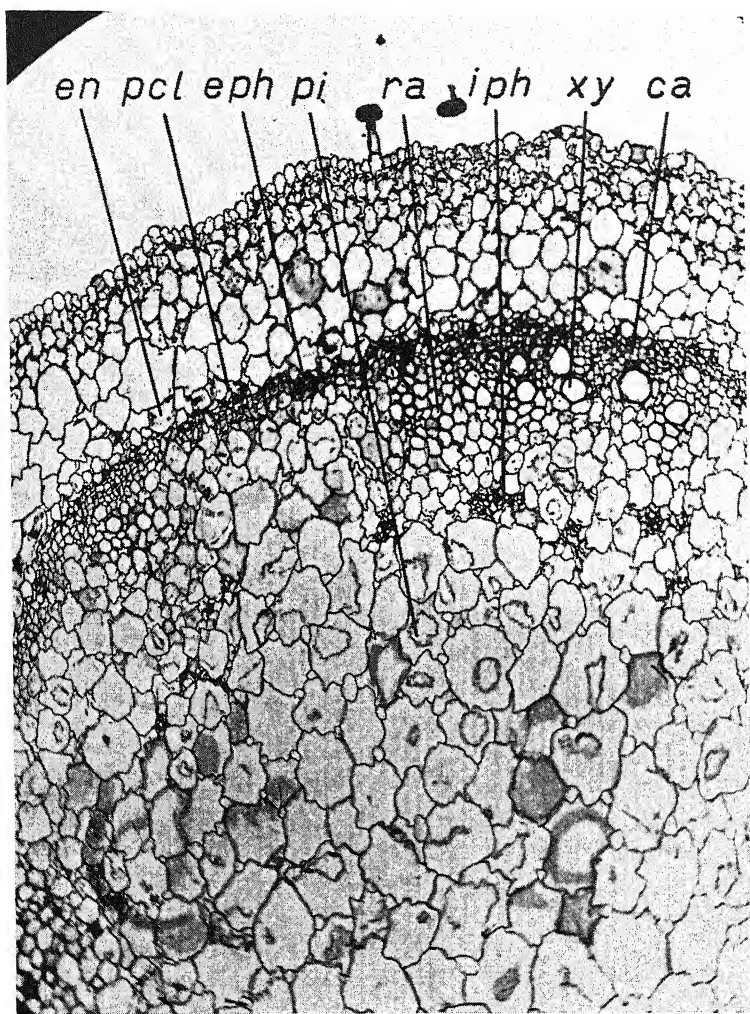


FIG. 2.—Transection through middle of third internode of tomato stem at time of application of indoleacetic acid, showing stages of development of various tissues. *en*, endodermis; *pcl*, pericycle; *eph*, external phloem; *iph*, internal phloem; *ca*, cambium; *xy*, xylem; *ra*, ray; *pi*, pith.

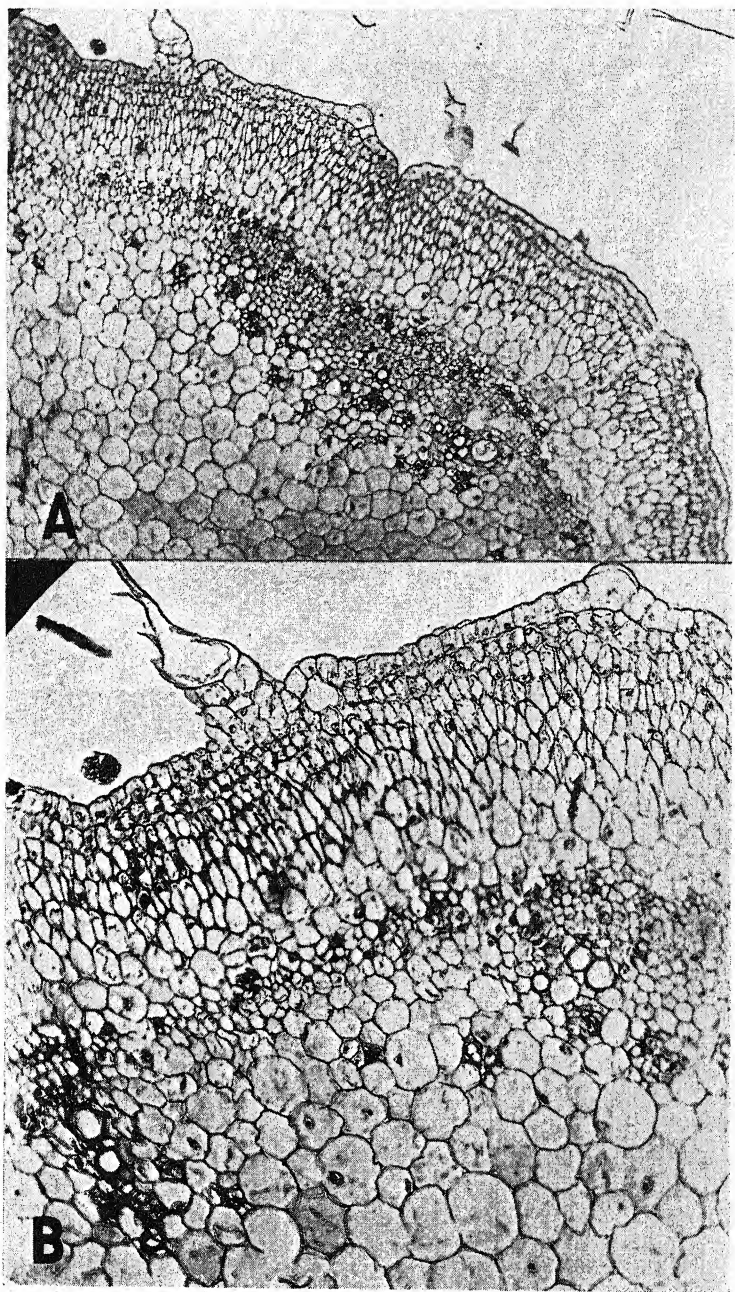


FIG. 3.—Twenty-four hours after treatment. *A*, section 400  $\mu$  from cut surface; *B*, sector of same enlarged. Epidermal cells slightly enlarged; not much division. Outer cortical cells appreciably enlarged and elongated radially. Endodermal cells enlarged. Other tissues show slight change.



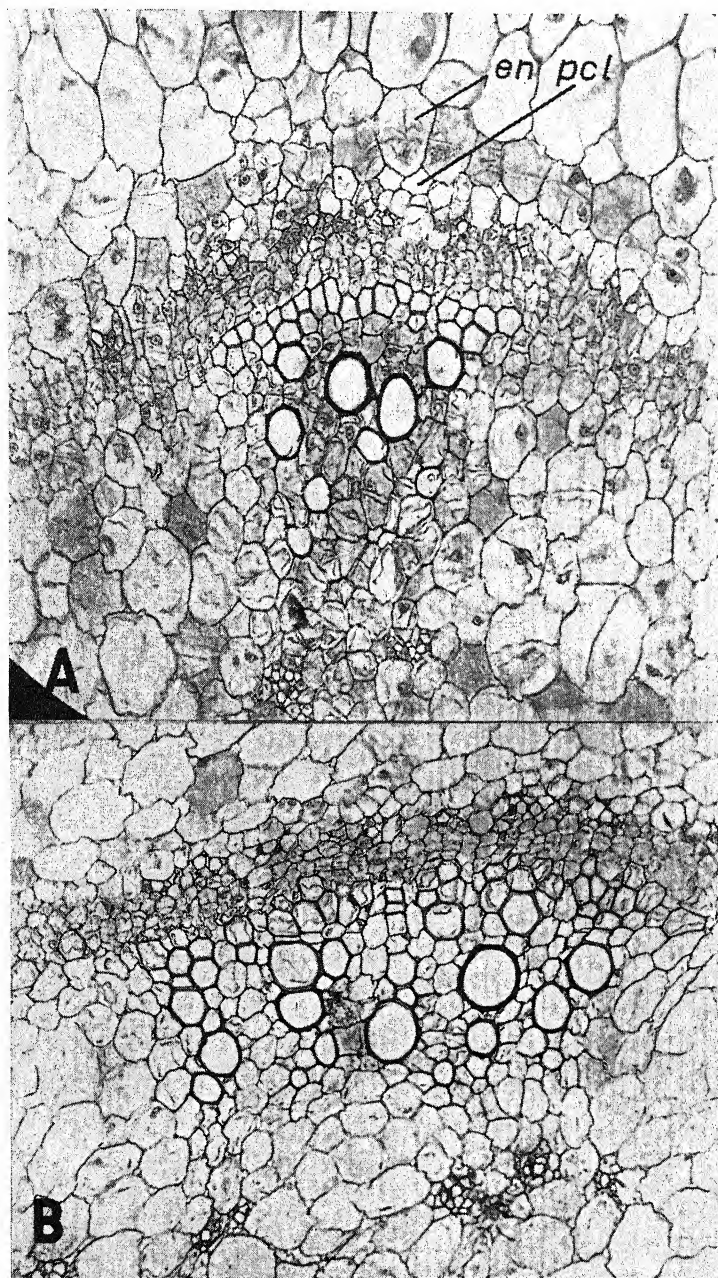


FIG. 4.—Fifty-four hours after treatment. *A*, section of bundle about 1.5 mm. below cut surface. Endodermal cells divided tangentially and radially. Occasional divisions in other cortical cells but not in pericycle. Parenchymatous cells in primary xylem, pith, and near internal phloem actively dividing. Divisions in external phloem frequent and in all planes. Slight differentiation of tissues from the cambium. *B*, 3 mm. below cut surface. No divisions in any tissue except the cambium.



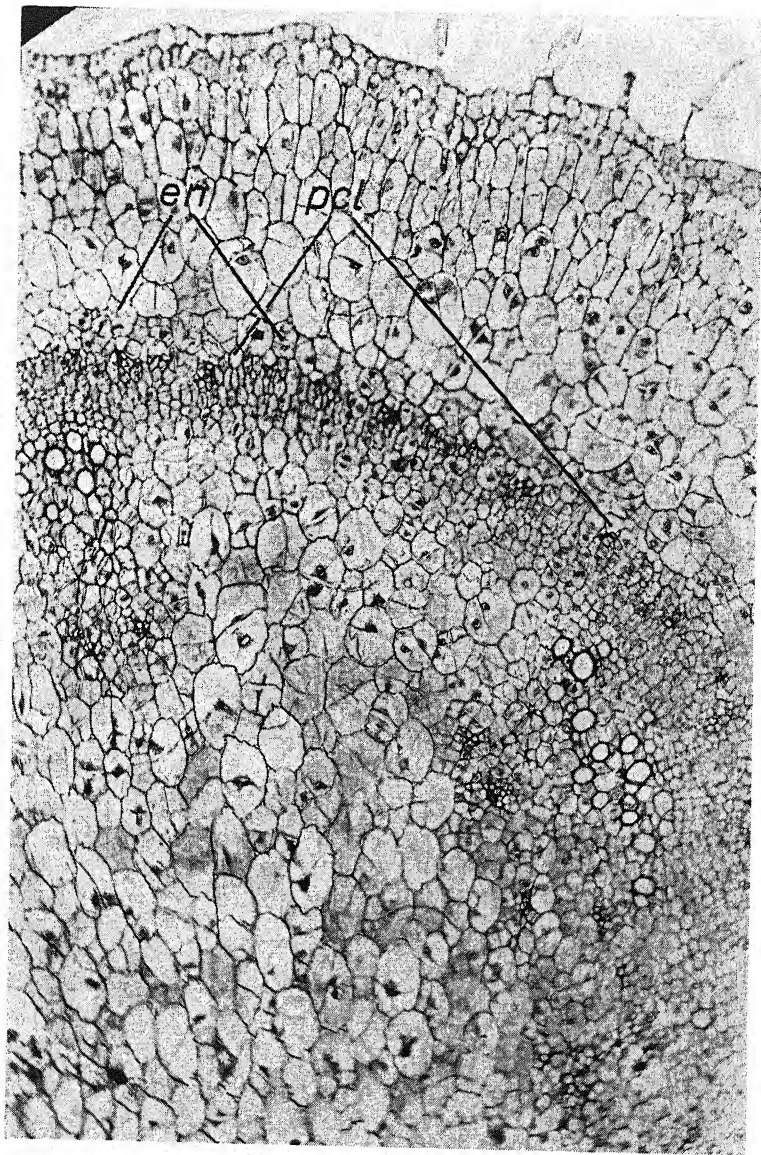


FIG. 5.—Sixty hours after treatment; about 1 mm. from cut surface. Much activity in cortical parenchyma, endodermis, external and internal phloem parenchyma, xylem parenchyma, ray, and pith. Pericyclic fibers unchanged except more nearly mature.

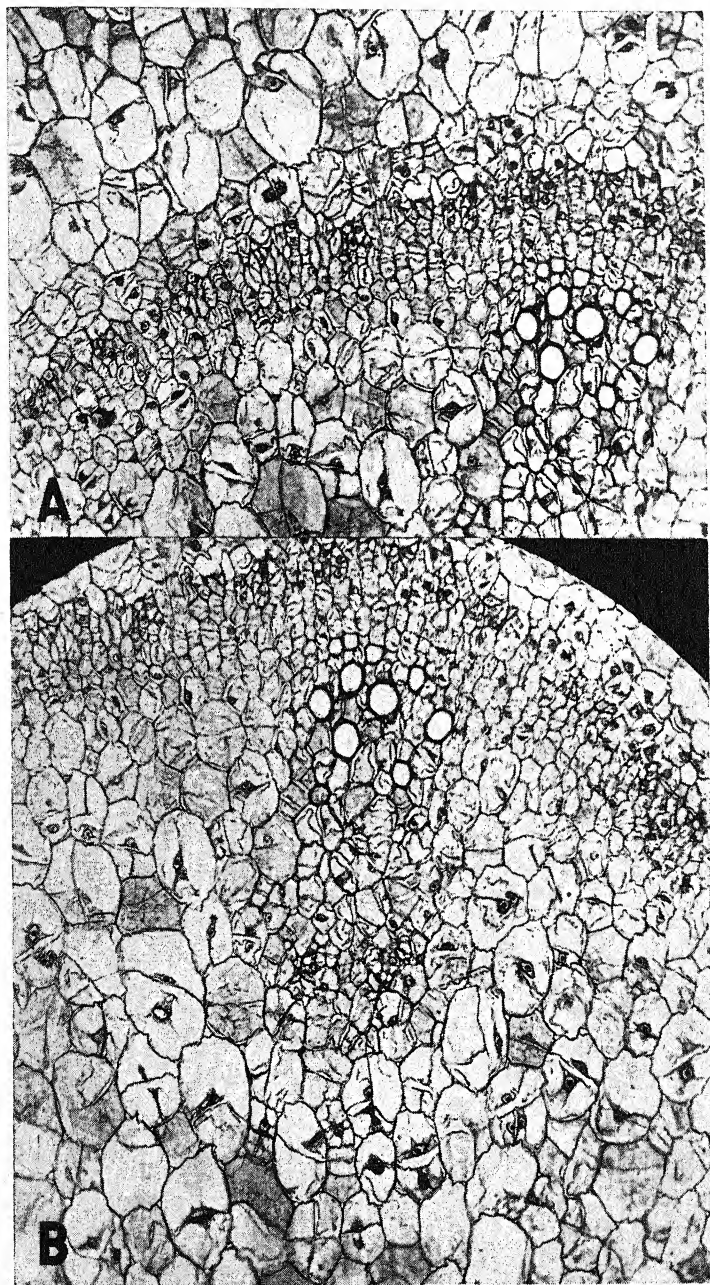


FIG. 6.—Same section as fig. 4 enlarged. *A*, details of outer region; *B*, of inner portions.

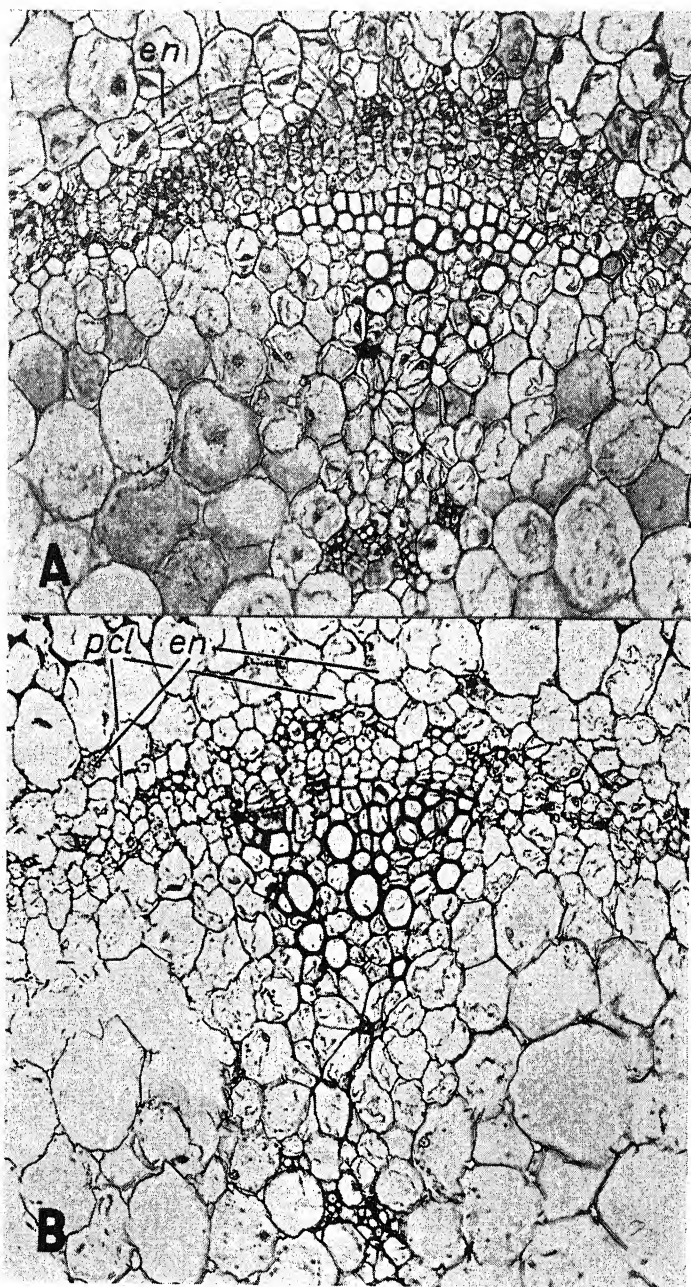


FIG. 7.—Sixty hours after treatment. *A*, approximately 1.5 mm. from cut surface. Meristematic activity confined to endodermis and external phloem. *B*, about 3 mm. below cut surface. Meristematic activity confined to cambium only. Other tissues approaching maturity.

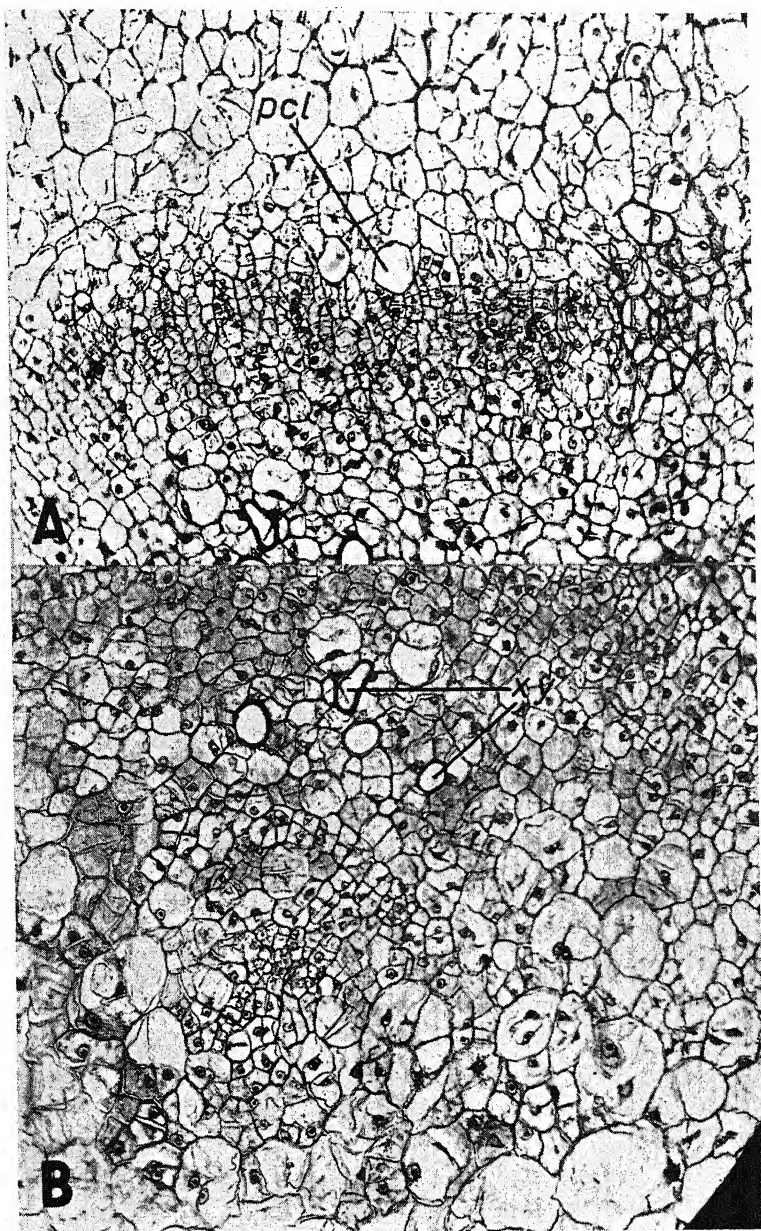


FIG. 8.—Eighty-four hours after treatment. *A*, approximately 200  $\mu$  below cut surface. Meristematic activity in cortical parenchyma and endodermis. Pericycle cells more nearly mature. Outer phloem parenchyma highly meristematic. Ray parenchyma at right of center with many divisions. *B*, same section. Meristematic activity of xylem, internal phloem, and pith.



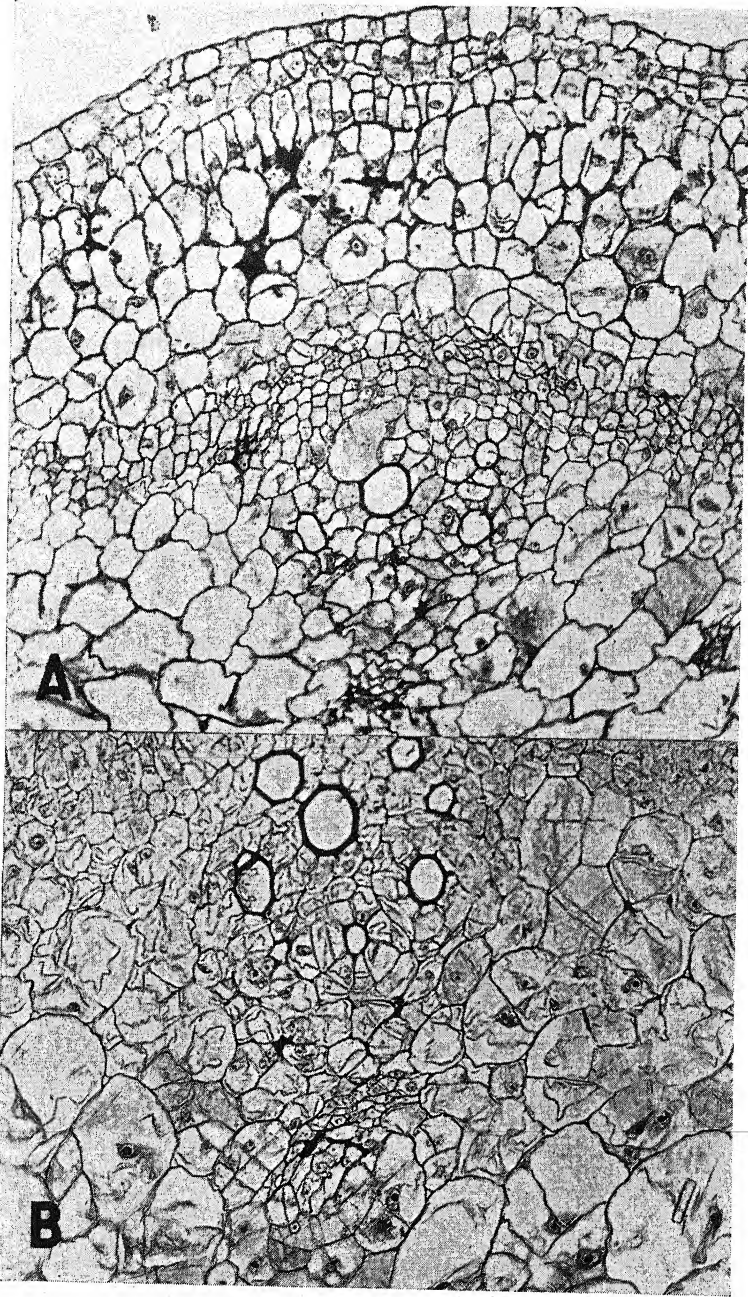


FIG. 9.—Ninety-six hours after treatment. *A*, section  $200\ \mu$  below cut surface, above region of greatest activity. Endodermis meristematic. *B*, about  $750\ \mu$  below cut surface, same bundle as in *A*. Great meristematic activity of all parenchymatous tissues.

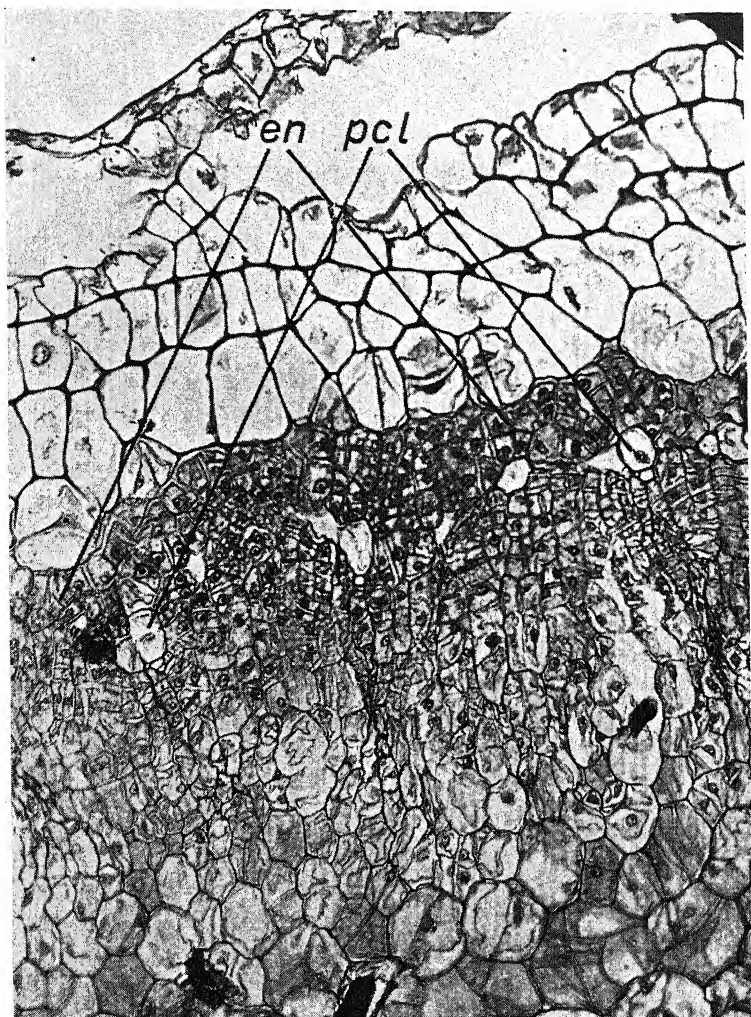


FIG. 10.—Ninety-six hours after treatment. Section through a ray at level slightly lower than fig. 8*B*. Endodermal derivatives in a band several cells wide. Some pericyclic cells nearing maturity, others crushed. Phloem parenchyma highly active.

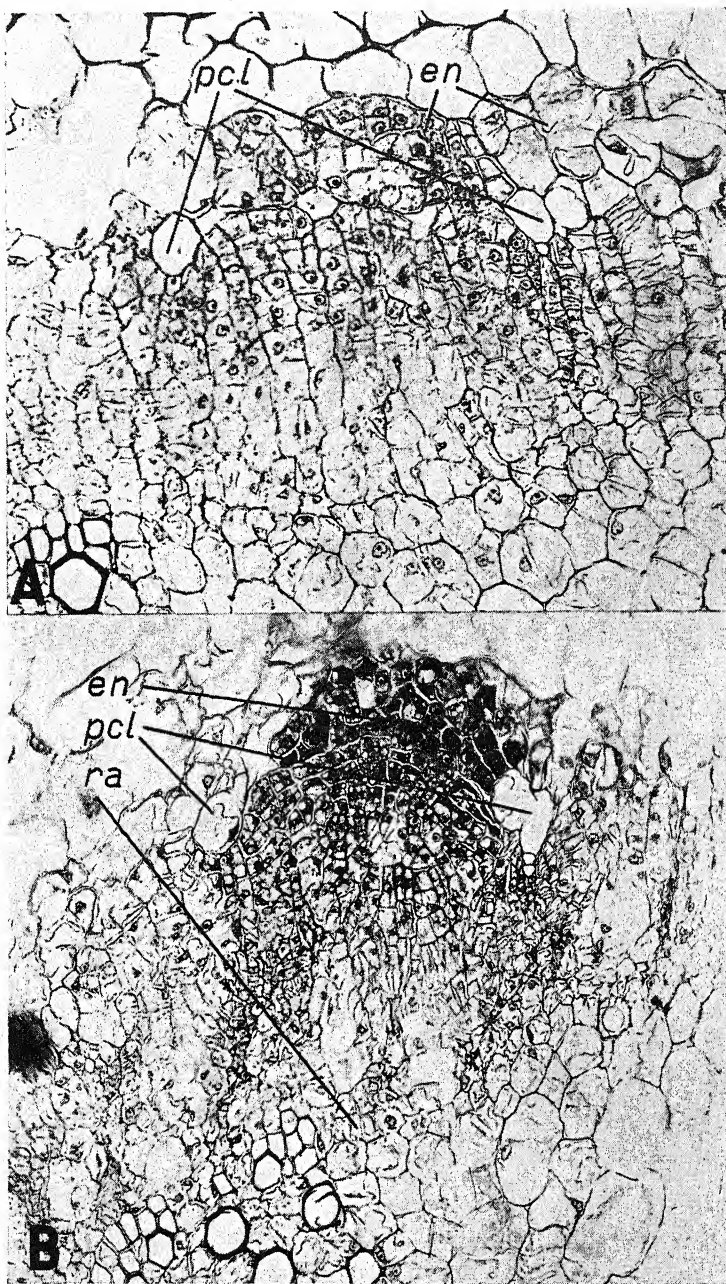


FIG. 11.—Ninety-six hours after treatment; 1.4 mm. below cut surface. *A*, endodermal derivatives constitute a band 3-5 cells wide. Pericycle cells nearing maturity or crushed. Phloem parenchyma and ray cells highly meristematic. *B*, detailed view showing relation of adventitious roots and ray. The cap over primordium of adventitious root derived from proliferated endodermis.

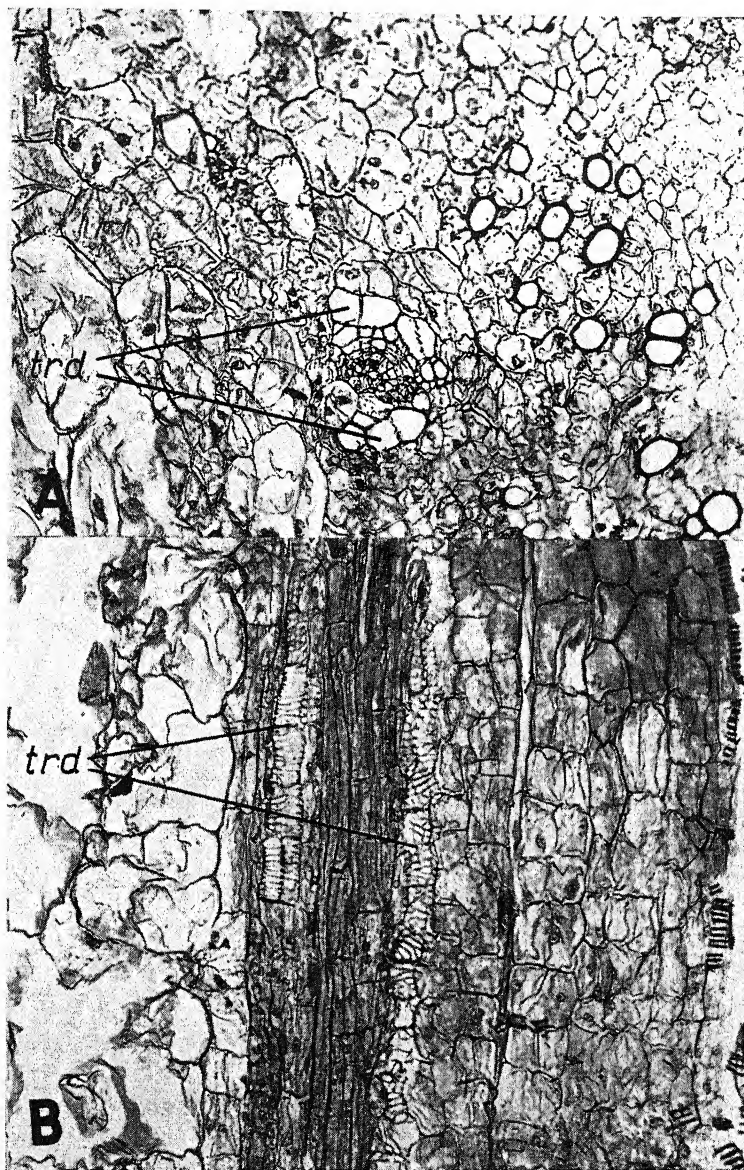


FIG. 12.—One hundred eight hours after treatment. Detail of region of internal phloem. *A*, cross section 1.2 mm. from cut surface. At center, group of sieve tubes and companion cells of internal phloem surrounded by tracheids derived from surrounding parenchymatous cells. *B*, longitudinal section. At left of center, strand of sieve tubes and companion cells with adjacent tracheids derived from surrounding parenchymatous cells as in *A*.



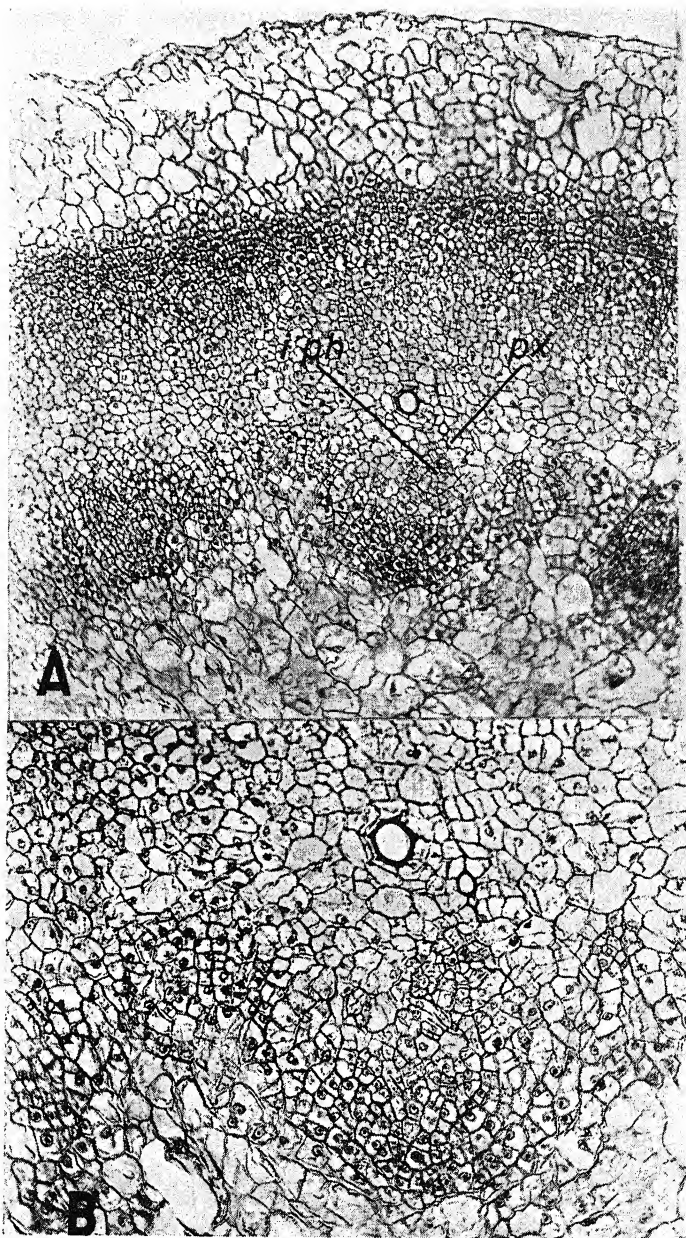


FIG. 13.—One hundred thirty-two hours after treatment;  $400\ \mu$  below cut surface. *A*, all parenchymatous tissues centripetal to inner cortex actively meristematic. Beginnings of three roots in vicinity of internal phloem. *B*, enlarged central portion of *A*.

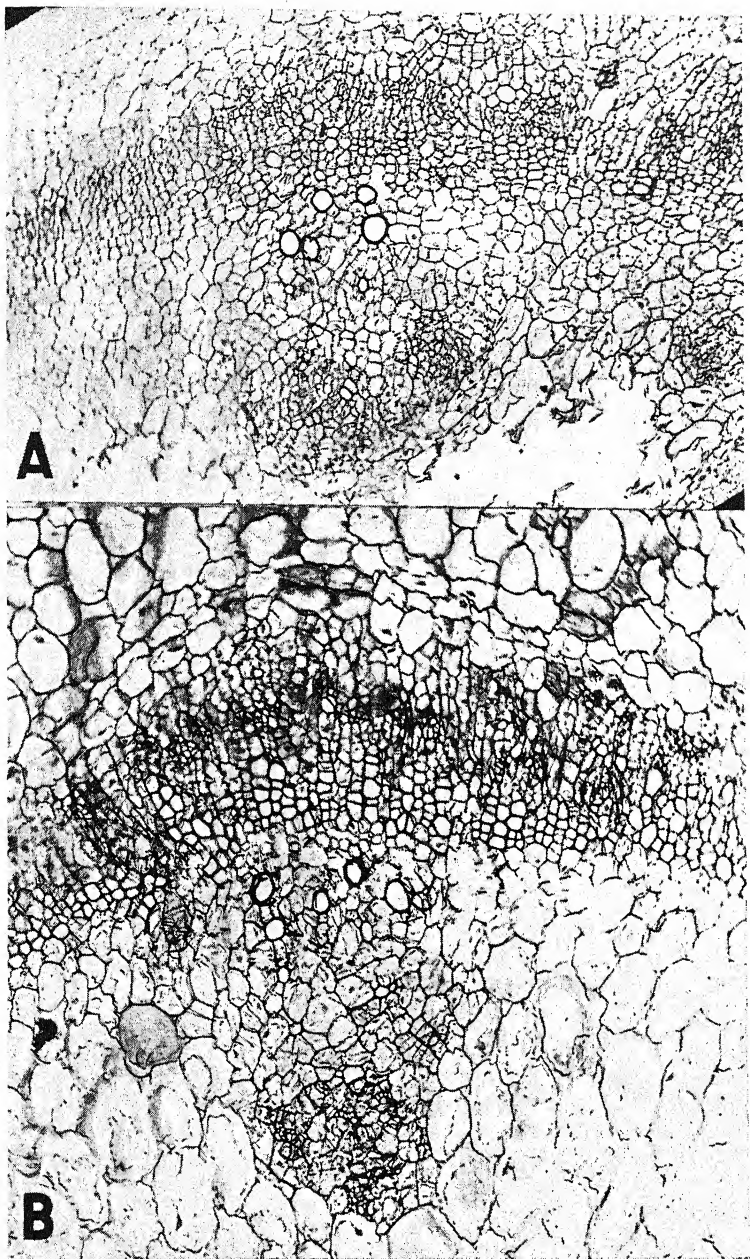


FIG. 14.—One hundred thirty-two hours after treatment. *A*, 700  $\mu$  from cut surface. Some of derived cells in region of internal phloem have matured as tracheids. Same stem as fig. 12. *B*, 1.8 mm. from cut surface. Same bundle as shown in *A*, slightly more enlarged. Greatest activity in region of pith adjacent to primary xylem and internal phloem. Fewer tracheids in region of internal phloem. Endodermis and external phloem also show proliferation.

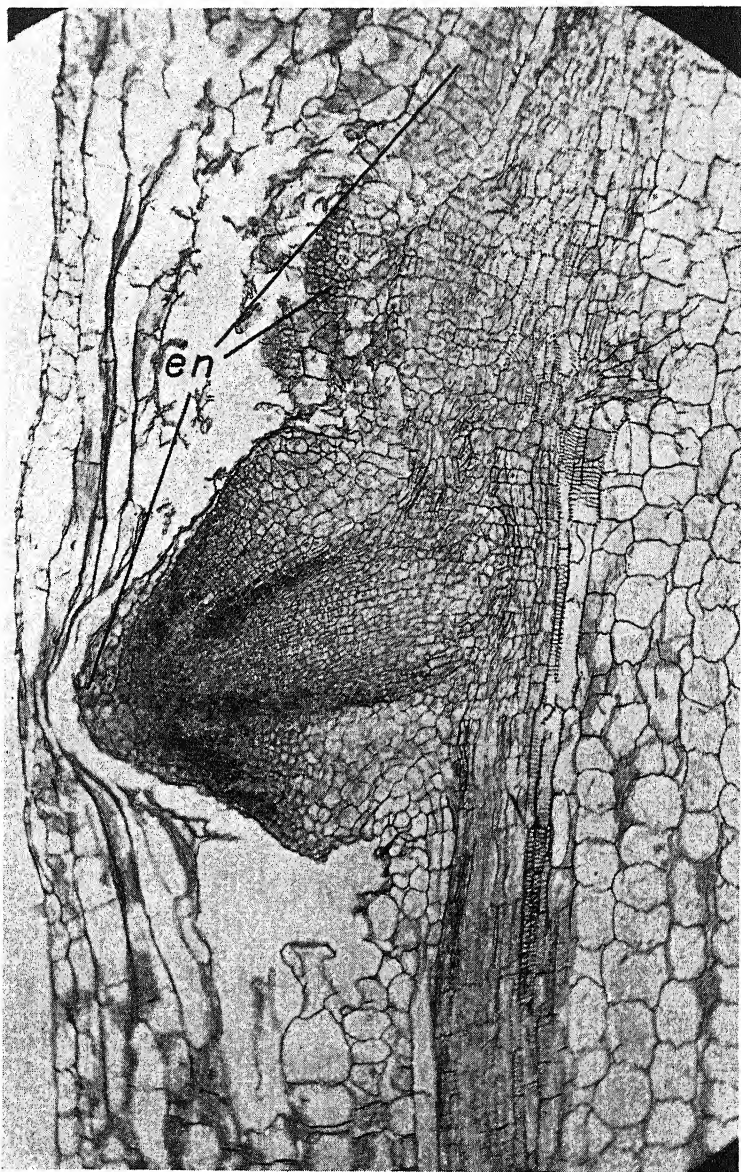


FIG. 15.—One hundred forty-four hours after treatment; median longitudinal section through adventitious root. Endodermis above root several cells in width. A portion of it has been broken away and caps the tip of root. Phloem also proliferated. There has been relatively little activity below the root.

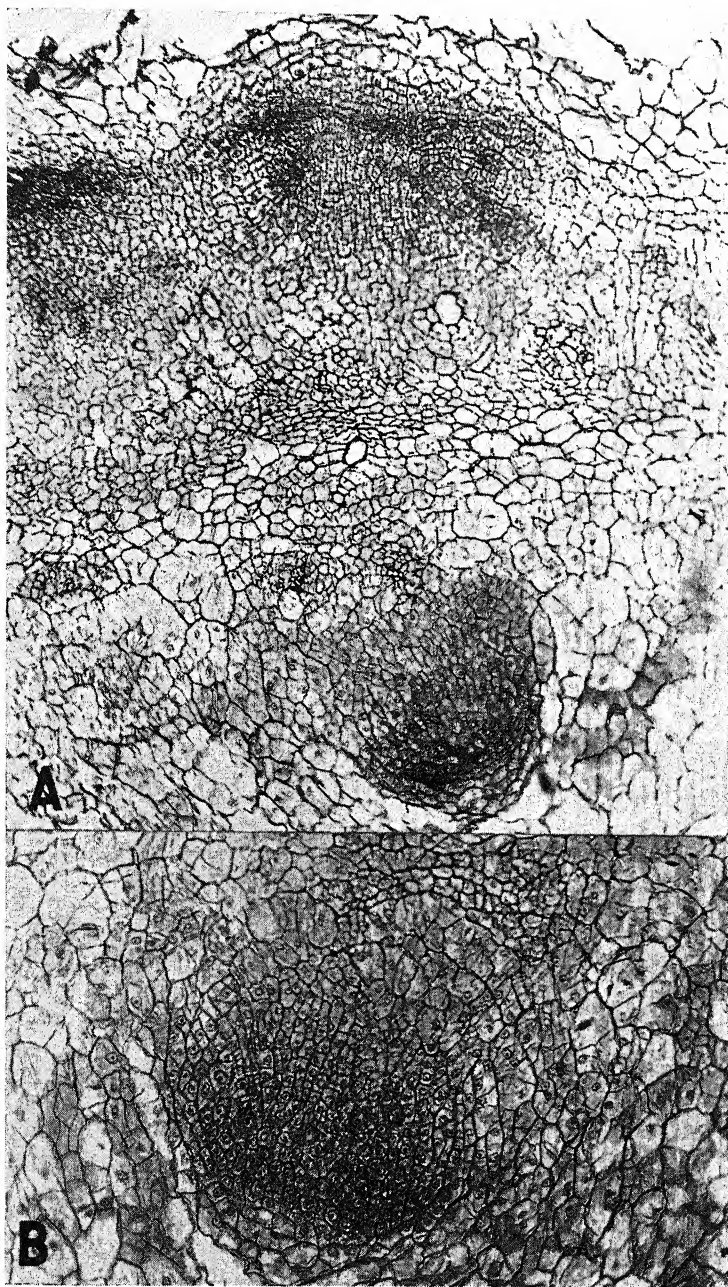


FIG. 16.—One hundred sixty-eight hours after treatment;  $600\ \mu$  below cut surface. *A*, adventitious roots derived from vicinity of external and internal phloem. *B*, enlarged view of young internal root.



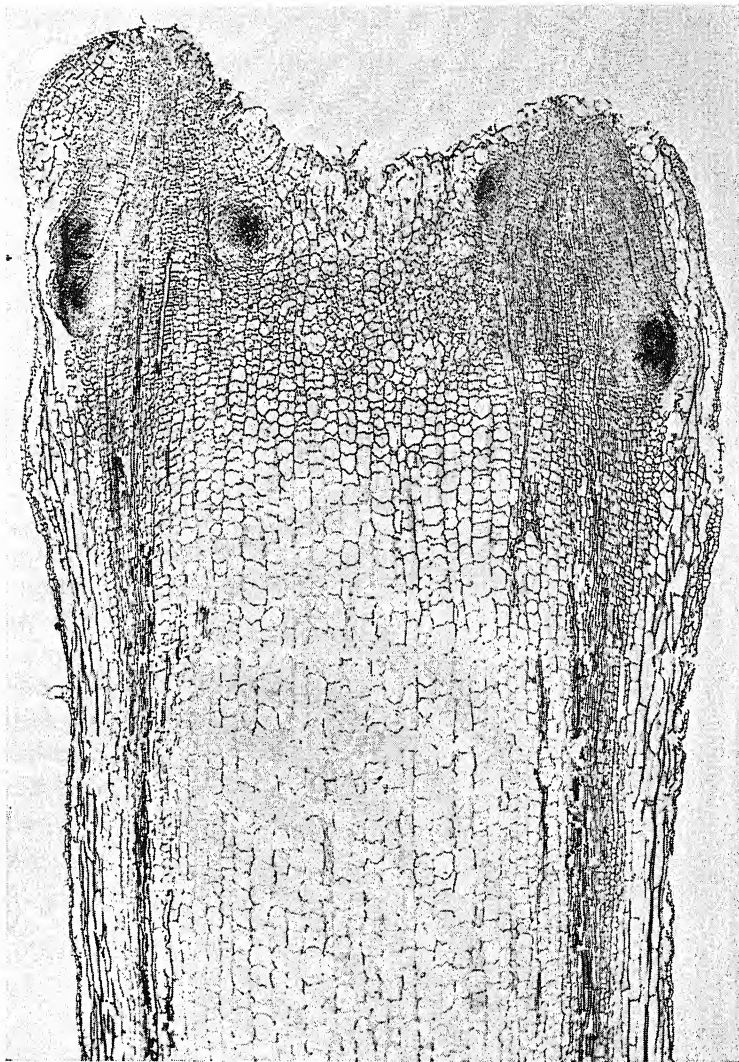


FIG. 17.—Two hundred sixteen hours after treatment; nearly median longitudinal section. Beginnings of both internal and external adventitious roots, above which there has been much greater proliferation of endodermis than below (*cf.* fig. 15). Pith has proliferated much more extensively several cells below treated surface than adjacent to it (*cf.* fig. 8).

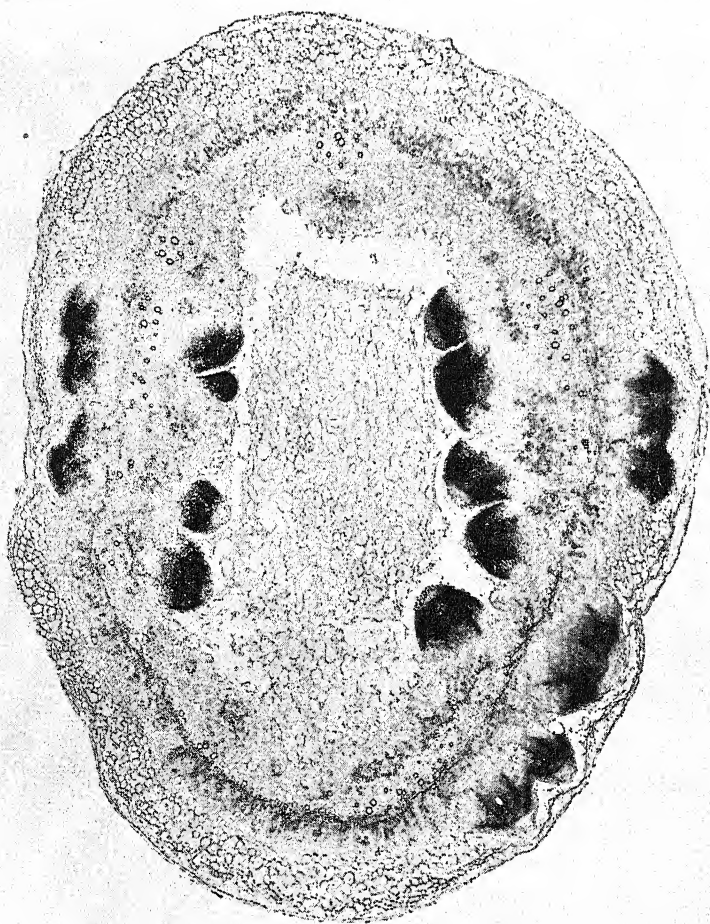


FIG. 18.—One hundred ninety-two hours after treatment; transection through zone of internal and external adventitious roots. Pith is disintegrating. It has proliferated somewhat in this region but not so much as farther down the stem (*cf.* fig. 17).

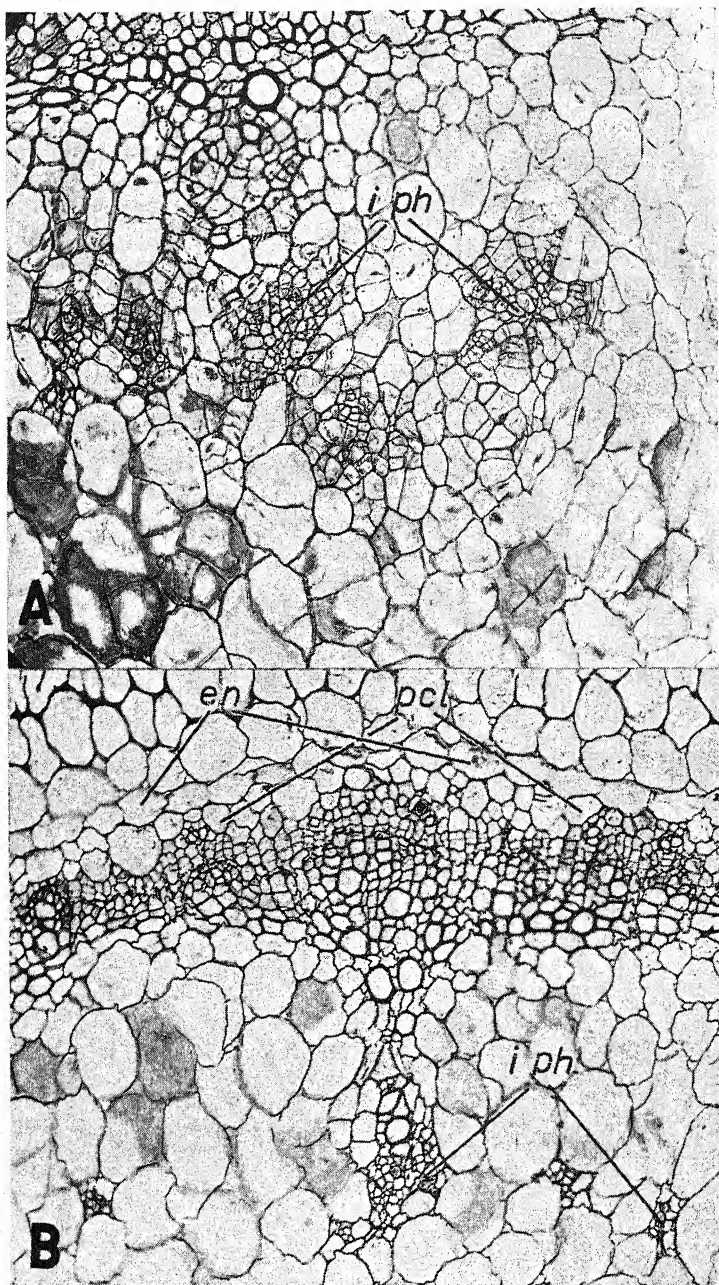


FIG. 19.—Two hundred sixteen hours after treatment. *A*, section 1.8 mm. below treated surface. Parenchymatous cells in vicinity of sieve tubes and companion cells of internal phloem highly meristematic. *B*, 4.0 mm. from treated surface, same stem. Cambium active but proliferation of other tissues slight. Some derivatives near internal phloem differentiated as tracheids.

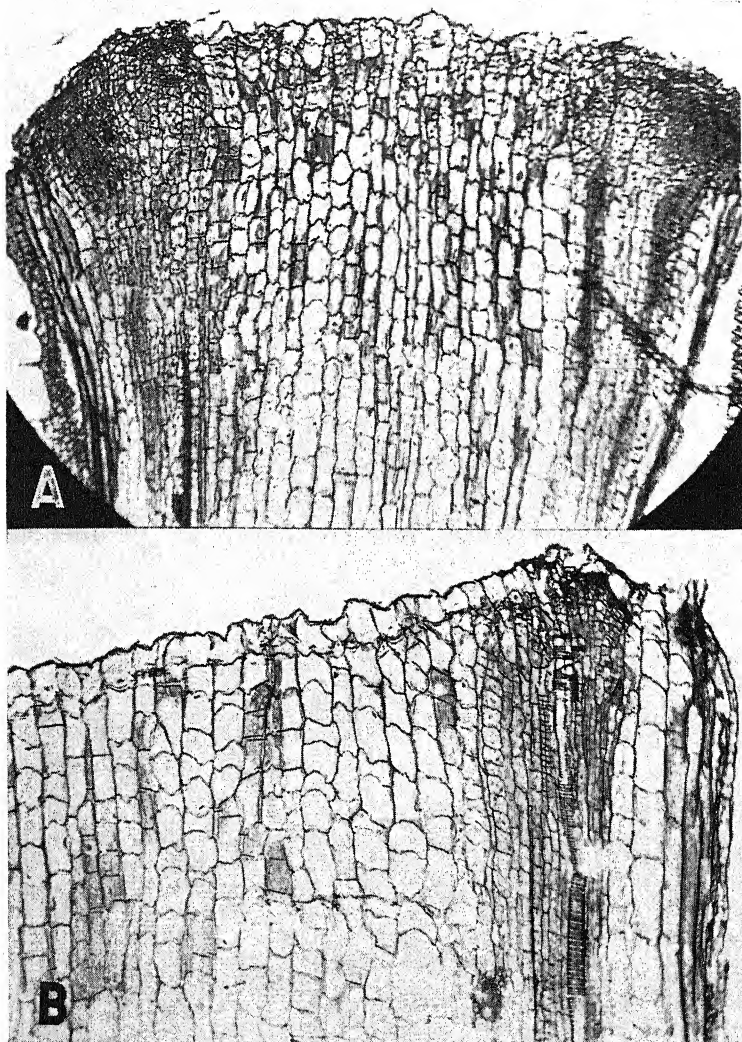


FIG. 20.—Twenty-three days after decapitation. *A*, cut surface untreated. Near it the phloem has proliferated, resulting in slight swelling of tip of stem. *B*, cut surface treated with lanolin only. A phellogen has developed across entire cut surface. Very little proliferation of other tissues except external phloem near the top.



STARCH.—The localization of starch in the treated stems was followed in detail during the experiment. Starch was concentrated in the endodermis and in the parenchymatous cells surrounding the xylem, and was abundant in the cortex and pith before any treatment was begun. The concentration of starch decreased following decapitation, regardless of the treatment. During the first 24 hours the stems that were treated with the indoleacetic acid mixture contained more starch than the controls which were treated with lanolin only. Following this period, the starch began to disappear in the cells surrounding the xylem in the treated stems. As time went on, in the plants treated with indoleacetic acid the starch began to decrease in the endodermis and in the pith cells which had become meristematic, but remained in those which were not dividing. As the tumors enlarged the concentration of starch decreased greatly in the proliferated endodermis. Immediately below the enlarged portion the starch was very abundant. The tumors were practically devoid of starch after 216 hours except for a layer three or four cells thick directly below the original cut surface. The cortical, endodermal, and pith cells in this layer retained their starch content throughout the experiment. Those treated with lanolin only contained abundant starch in the endodermis, cortex, and pith, except for a region three or four cells below the cut surface. Stems which were decapitated only were similar to the lanolin controls except that the starch was present in the cells up to the cut surface. Apparently the capacity of cells at the cut surface to digest starch was destroyed by the indoleacetic acid, since the lanolin controls contained no starch in a comparable area.

The endodermis adjacent to the young roots became completely void of starch. The root caps of all young roots contained abundant starch at first; this, however, soon disappeared.

#### Comparative responses of bean and tomato

The histological reactions of the tomato in response to indoleacetic acid are in many respects similar to those of the bean (1). In both the tissues of the cortex, phloem, rays, and pith become meristematic in response to treatment but the degree of the response differs. Moreover, the presence of an active internal phloem in tomato, in contrast

to the bean, results in further differences, which may be correlated with structure.

The degree of activity, tissue for tissue, is less in the tomato than in the bean, and the zone of activity does not extend so far down the stem. In the bean, vascular strands which resembled the tumor strands reported by SMITH, BROWN, and McCULLOCH (2) were found in the endodermis and phloem. The only evidence of structures similar to these in the tomato was found arising from the internal phloem (fig. 12).

In the bean enormous tumors are produced, largely through long continued activity in the pith. Gross observations were made on several hundred tomato tumors over a period of two months, and under the conditions of these experiments little, if any, further growth was observed after the first ten days.

In the bean a ring of adventitious roots is formed as a result of activity in the phloem and rays. In the tomato two rings of roots are formed, the outer being similar in origin and development to that of the bean. The roots of the inner ring arise centripetal to and in close association with the internal phloem, and grow into the pith.

Many studies have shown that treatment with indoleacetic acid results in the formation of adventitious roots, in many cases on stems that would not ordinarily form such roots. The investigations on bean and tomato have shown that, in these two plants at least, treatment will induce the formation of such roots in tissues that ordinarily do not produce them. In both plants treatment with indoleacetic acid has resulted in the formation of adventitious roots from derivatives of the phloem or rays.

In the tomato adventitious roots are formed also from derivatives of the internal phloem and pith. The fact that indoleacetic acid induces formation of adventitious roots in tissues other than the pericycle indicates that its action is not merely a speeding up of the normal process of adventitious root formation. The roots formed in the external phloem, under favorable conditions, grow out through the cortex and have the appearance of adventitious roots formed on untreated plants. So far the roots formed from the internal phloem and pith have been observed to grow into the pith a short distance and then cease further extension. Whether these roots could be in-

duced to continue their growth by some type of treatment of the stem is not yet known. Structurally they have the appearance of roots formed anywhere else in the plant. This indicates that the treatment with indoleacetic acid has not fundamentally altered the hereditary make-up of the individual cells of the plant. It is obvious that the usual course of development of some of the tissues is altered, but no types of cells or types of tissues not customarily formed were differentiated. Such differences as exist are those of degree, rather than of kind. Generalization as to causes in relation to the developmental patterns resulting from treatment with indoleacetic acid must await further cytological and histological investigation.

### Summary

1. Tomato seedlings were decapitated above the second lobed leaf and a mixture of 20 mg. of indoleacetic acid in 1 gm. of lanolin was applied to the cut surface. Similarly, decapitated plants were kept as controls, either untreated or with pure lanolin applied to the cut surface.

2. Gross observations and histological and microchemical studies were made of treated and control material collected at frequent regular intervals for a period of 216 hours after treatment.

3. The internode terminated by the cut surface fails to enlarge after decapitation unless the cut surface is treated with indoleacetic acid-lanolin mixture. The internodes so treated enlarge at about the same rate as do the internodes below.

4. The first change observed after treatment was enlargement of cells of the epidermis and cortex.

5. Many of the tissues of the stem became meristematic in response to the treatment, although most of this activity was confined to a zone 0.5 to 2 mm. from the treated surface. Most parenchymatous cells in this zone underwent some divisions; the endodermal cells, external and internal phloem parenchyma, and pith cells adjacent to the internal phloem exhibited the greatest activity. There were, however, certain tissues comprising a small portion of the stem whose living cells showed little or no meristematic activity. These include epidermis, most of the pericycle, sieve tubes and companion cells, and internal fibers.

6. A ring of adventitious roots was formed around the stem as a result of activity in the external phloem. Portions of the active band of endodermal cells covered the tips of these roots as they pushed through the cortex. Another ring of adventitious roots was formed near the pith as a result of activity of cells of the internal phloem and adjacent pith.

7. Nitrates were more concentrated in the controls than in the stems treated with indoleacetic acid. The concentration of protein in the treated stems increased in those areas where cell divisions became most abundant. Starch disappeared from the cells of the treated stems as the tumors enlarged, but remained in the controls.

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# EFFECT OF EXTRACTS FROM THE CORN PLANT ON GROWTH OF EXCISED ROOT TIPS<sup>1</sup>

WILLIAM J. ROBBINS AND VIRGINIA B. WHITE

(WITH THREE FIGURES)

## Introduction

In the experiments performed in this laboratory on the cultivation of excised corn root tips under sterile conditions, extracts of corn grains and other parts of the corn plant have occasionally been used to supplement the mineral solution and dextrose used as a nutrient medium. The corn grain is the natural source from which a successful medium for the growth of excised roots should be derived, since the root attached to the grain may secure from that source everything it requires for early growth, save water and oxygen. The earlier experiments, however, indicated that extracts from the grain or from the corn top were of little benefit or were injurious (4). In the present paper results are reported on the effect (1) of milk from young grains, (2) of the diffusate into agar from germinated grains, (3) of the diffusate into agar from root tips and shoot tips, (4) of root extracts, (5) of extracts of germinated grains, and (6) of experiments in which the solution containing various supplements was renewed during the growth of the roots.

**MATERIALS AND METHODS.**—The materials used and methods employed have been described in an earlier paper (7) and will not be repeated here.

## Experimental results

**MILK FROM YOUNG CORN GRAINS.**—In the few experiments performed, milk from corn in the roasting ear or soft milk stage was toxic at the higher concentrations and ineffective at lower concentrations, at least in an agar medium.

In one experiment, roots originally 2 mm. long were grown in 125 cc. Erlenmeyer flasks on qualitative filter paper moistened with unheated milk from corn grains. In one series the undiluted milk was

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used; in the other, milk diluted ten times with redistilled water. Because of the difficulty in securing the milk in a sterile condition, the series were not large. However, the milk at the concentrations used was decidedly injurious to the excised root tips.

In a second experiment the milk was added at different concentrations to solution C<sup>2</sup> plus 0.5 per cent agar and sterilized. At a concentration equivalent to the milk from 1.5 or 3 grains per flask the milk was toxic; at concentrations from 0.5 grain or less no effect on the growth of the roots was noticed.

**DIFFUSATE FROM GERMINATED CORN GRAINS.**—The diffusate from a germinated grain was beneficial to the growth of root tips less than 1 mm. in length in solution CF plus agar.

The experiments were carried out as follows. Corn was germinated under sterile conditions, and when the seedling root was about 7 cm. long both root and shoot were removed. The grain was cut longitudinally in half at right angles to the flat surface, and each half was transferred to a sterile petri dish containing a layer of agar. The half grain was placed in the center of the dish and the cut surface pressed firmly down against the agar. By testing the agar at different distances from the grain with Fehling's solution, the xanthoproteic reaction, and Millon's reagent, it was found that reducing sugar diffused from the grain to the edge of the dish in three days; the amino acids, in six days. This was taken to indicate that whatever diffuses from the grain would probably be generally distributed through the agar in a petri dish within seven days after the split grain was placed therein.

Plates of CF agar were prepared and in some of them a half grain was placed. After seven days excised root tips were placed in the plates, which were incubated at room temperature in the dark. In some cases the half grain was removed before the roots were placed in the dishes; in other cases the grain was left in the dish. The growth of the roots was measured daily and the experiments were limited to ten days or two weeks because of the drying out of the agar in the

<sup>2</sup> Solution C contained calcium nitrate, 50 p.p.m.; magnesium sulphate, 10 p.p.m.; potassium dihydrogen phosphate, 10 p.p.m.; ferric chloride, 1 p.p.m.; dextrose, 2%. Solution CF consisted of solution C plus manganese chloride, 0.1 p.p.m.; zinc chloride, 0.1 p.p.m.; and sodium borate, 0.1 p.p.m.

dishes. Under such conditions a beneficial effect of the grain was found for roots less than 1 mm. in length; longer roots were not benefited although different results might have been found had conditions permitted the experiments to run for longer periods. A typical experiment is summarized in table 1.

DIFFUSATE FROM ROOT TIPS OR COLEOPTILE TIPS.—WENT (9) secured the growth hormone from coleoptile tips by permitting it to diffuse into agar. The effect of the diffusate from coleoptile tips and root tips on the growth of excised corn root tips was determined by

TABLE 1  
EFFECT OF DIFFUSATE FROM ONE-HALF CORN GRAIN ON  
GROWTH OF ROOTS LESS THAN 1 MM. IN LENGTH. GROWN 10  
DAYS IN PETRI DISHES AT ROOM TEMPERATURE IN DARK

MEDIUM	No. roots	AVERAGE ORIGINAL LENGTH (MM.)	AVERAGE FINAL LENGTH (MM.)
CF agar.....	14	0.60	6.07
CF agar plus half grain	18	0.69	12.56

the writers. The results were not definite although some beneficial effect was secured from the root tips and some detrimental effects from the coleoptile tips. The experiment was performed as follows.

Petri dishes containing solution CF plus 0.5 per cent agar were prepared and divided into three groups. In those of one group root tips 2 mm. long were placed on end with the cut surface pressed down into the agar. In a second group 2 mm. coleoptile tips were similarly placed. The third group was undisturbed. After four days the root tips and coleoptile tips were removed and on the spots formerly occupied by them excised root tips of less than 1 mm. original length were placed. Measurements of growth were made daily, the experiment being terminated after seven days. The curves giving the growth in mm. per day (fig. 1) and the final lengths (table 2) show that the growth in the plates with diffusate from the root tips was somewhat greater than the check, and that in the plates with diffusate from the coleoptile tips it was somewhat less than the check.

EXTRACTS FROM ROOTS.—It was thought that the roots themselves might contain some material which would be beneficial to the growth of excised root tips. Extracts of different portions of the terminal 15 mm. of the primary roots of young seedlings were used at various concentrations. In solution C plus agar the root extract was beneficial when added at a concentration of 0.1, 2.5, 3, 5, and 10 roots per flask containing 30 cc. of medium in which 1 or 2 mm. root

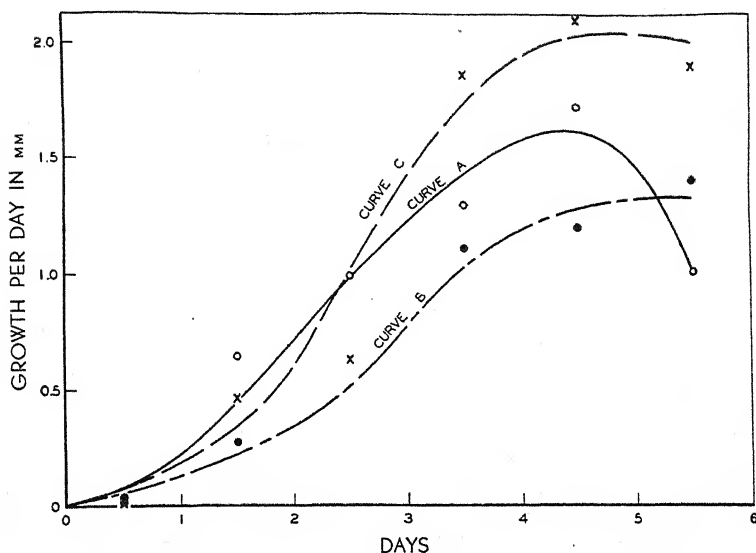


FIG. 1.—Growth in mm. per day of short root tips in solution C plus agar (curve A), solution C plus agar on which a coleoptile tip had been placed (curve B), and in solution C plus agar on which a root tip had been placed (curve C).

tips were grown individually. The benefit increased up to and including the extract of 5 roots; the effect of the extract from 10 roots was less marked. The extracts were made from 3 mm. portions of the roots. In some cases the extracts from the more basal portions of the tip were somewhat more beneficial than from those which included the tip itself.

The extracts were prepared by cutting the terminal 15 mm. of the primary roots of young seedlings into five 3 mm. portions. The segregated parts of the roots were heated to boiling in redistilled water



and macerated. The extract was filtered and filtrate added to the medium. Although qualitative filter paper was used in filtration, the amount of filter paper extract added per flask was too small to be significant. In table 3 the results of a single experiment with root tips 2 mm. in length are given.

TABLE 2

EFFECT OF DIFFUSATE FROM 2 MM. ROOT TIPS OR 2 MM. COLEOPTILE TIPS  
ON GROWTH OF SHORT EXCISED ROOT TIPS IN SOLUTION CF  
PLUS AGAR. PERIOD OF GROWTH 7 DAYS

TREATMENT	No. ROOTS	LENGTH			
		AVERAGE ORIGINAL (MM.)	AVERAGE FINAL (MM.)	MAXIMUM (MM.)	MINIMUM (MM.)
None.....	17	0.59	6.3	14.0	1.5
Root tips.....	21	0.62	7.6	22.0	1.0
Coleoptile tips.....	17	0.66	5.1	15.5	1.4

TABLE 3

EFFECT OF EXTRACT OF 3 MM. PORTIONS OF SEEDLING CORN ROOTS ON GROWTH  
OF EXCISED CORN ROOT TIPS. GROWN IN 30 CC. OF SOLUTION C PLUS 0.5 PER  
CENT AGAR IN 125 CC. ERLNMEYER FLASKS FOR 46 DAYS AT ROOM TEMPERA-  
TURE IN DARK. ORIGINAL LENGTH 2 MM.

ADDITION TO SOLUTION C PLUS AGAR	No. ROOTS	AVERAGE FINAL LENGTH (CM.)	AVERAGE NO. SEC- ONDARY ROOTS	MAXIMUM LENGTH (CM.)	MAXIMUM NO. SEC- ONDARY ROOTS	MINIMUM LENGTH (CM.)	MINIMUM NO. SEC- ONDARY ROOTS
None.....	11	17.0	66	44.4	116	4.1	10
Extract of 5 roots	9	28.5	99	56.0	189	8.5	28
Extract of 10 roots	11	23.0	52	67.4	153	4.1	7

EXTRACTS FROM CORN GRAINS.—Extracts of germinated corn grains were found to be injurious at higher concentrations and beneficial at lower concentrations.

The extracts were prepared from grains which had been germinated in dishes between sheets of filter paper. When the roots of the seedlings were from 10 to 15 cm. long, the shoots and roots were removed and the grains ground in a mortar. The ground material was

covered with about three times its volume of redistilled water and after 24 hours at room temperature the liquid was filtered through several layers of washed cheesecloth. The filtrate was used in the experiments.

When extract equivalent to that from one grain or two grains was added to 30 cc. of 2 per cent glucose or of solution C, distinct injury was produced. Root tips originally 1 cm. long made little growth, the roots swelled, the cortex wrinkled in ridges perpendicular to the long axis of the root and in some cases split away from the central cylinder. The apical meristem did not swell materially. A microscopic examination of some of the roots after 30 days in the solutions showed the cortical cells very much enlarged, some of them 0.3 mm. in diameter and many of them still alive. Enlargement had occurred to within 1 mm. of the root tips. Whether the apical meristem was still alive could not be determined by microscopic examination. Some of the cultures containing the grain extract were diluted with sterile solution C at the end of four weeks and in about one week new growth appeared at the root apex. The new tip was slender and grew out through the tissues of the old tip, representing probably the regeneration of a new tip from the pericycle. The maximum new growth made was 1 cm.

While not encouraging from the standpoint of the cultivation of excised root tips, these results are of interest because the injury is similar in character to that found in abnormal roots grown in solution C and because of the hypertrophy of the cortical cells, which remained alive for four weeks.

When used at a concentration equivalent to the extract of 0.1 of a grain per 30 cc. of medium in which an individual root was grown, beneficial effects were found as illustrated by the following experiment:

Roots originally 1, 2, 3, 4, or 5 mm. long were grown individually in 30 cc. of solution C plus 0.5 per cent agar or the same medium plus grain extract in 125 cc. Erlenmeyer flasks, for 70 days at room temperature in the dark. The growth was measured at intervals during the first 20 days. The results are summarized in table 4.

The total growth in solution C plus agar was approximately proportional to the length of the original tip. This is shown in table 5

where the growth per mm. of original length is given. These results are contrary to those secured in the modified Pfeffer's solution and previously reported (4) where root tips varying from an average original length of 0.35 to 6.06 cm. reached approximately the same average final length. Apparently the growth in the latter solution was limited by the medium. Since the average growth in solution C plus agar was proportional to the original length of the root tip, it

TABLE 4

EFFECT OF GRAIN EXTRACT ON GROWTH OF EXCISED CORN ROOT TIPS ORIGINALLY 1, 2, 3, 4, OR 5 MM. LONG GROWN 70 DAYS IN 30 CC. OF SOLUTION C PLUS 0.5 PER CENT AGAR IN 125 CC. ERLLENMEYER FLASKS AT ROOM TEMPERATURE IN DARK

ADDITION	ORIGINAL LENGTH (MM.)	NO. ROOTS	AVERAGE FINAL LENGTH (CM.)	AVERAGE NO. SECONDARY ROOTS	MAXIMUM LENGTH (CM.)	MAXIMUM NO. SECONDARY ROOTS	MINIMUM LENGTH (CM.)	MINIMUM NO. SECONDARY ROOTS
None.....	1	11	5.3	21	24.5	106	1.5	1
Grain extract...	1	12	11.6	45	41.5	206	1.3	0
None.....	2	14	14.4	65	47.1	220	4.2	12
Grain extract...	2	11	17.9	59	37.9	122	3.2	10
None.....	3	12	19.1	84	36.8	163	8.9	44
Grain extract...	3	14	22.3	81	53.8	206	6.6	18
None.....	4	11	27.0	128	58.1	236	9.3	42
Grain extract...	4	15	25.5	98	77.4	296	9.3	25
None.....	5	9	29.6	124	51.0	220	17.0	74
Grain extract...	5	14	29.5	245	68.0	238	17.0	73

would seem that the growth in that medium was limited by materials originally contained in the root tip. The grain extract supplemented these materials and permitted greater growth of root tips originally 1, 2, or 3 mm. long to occur. The grain extract did not affect the growth of tips of longer original length, probably because the experiments were not continued long enough to exhaust the larger amount of material contained in the longer root tips.

The effect of the grain extract in relation to the original length of the root tips may be calculated in the following way. Assuming that the average growth per mm. of length of the original root tips in the agar medium without grain extract is 63 mm. (table 5), then the effect of the grain extract on the 1 mm. root tips was to produce 116

minus 63 or 53 mm. additional growth. For the 2 mm. root tips the effect of the grain extract similarly calculated was 179 minus 126 or 53 mm.; for the 3 mm. root tips, 34 mm.; for the 4 mm. root tips, 3 mm.; and for the 5 mm. root tips, -20 mm.

No evidence can be deduced from these experiments as to whether the deficiency in the modified Pfeffer's solution supplied by the agar medium or the deficiency in the root supplied by the grain extract is organic or inorganic, qualitative or quantitative.

TABLE 5  
GROWTH PER MM. OF ORIGINAL LENGTH OF ROOT  
TIPS GROWN IN 30 CC. OF SOLUTION C PLUS  
0.5 AGAR OR IN SAME MEDIUM PLUS GRAIN EX-  
TRACT (SEE TABLE 4)

ORIGINAL LENGTH (MM.)	GROWTH PER MM. ORIGINAL LENGTH	
	CHECK (MM.)	GRAIN EXTRACT (MM.)
1.....	53	116
2.....	72	89
3.....	64	74
4.....	67	64
5.....	59	59
Average.....	63	80

Further evidence on the effect of extracts on the growth of excised roots was secured by another procedure.

EFFECT OF ADDITIONS TO SOLUTION C AND OF CHANGE OF SOLUTION.—In a preliminary experiment root tips of 1 cm. original length were grown in 125 cc. Erlenmeyer flasks containing 40 cc. of 2 per cent dextrose and the extract of 0.1 or 0.01 of a corn grain. By marking the position of the tip of the main root on the outside of the flask by means of a wax pencil, the daily growth of individual roots was recorded at intervals. When the growth of a particular root decreased to 0.5 cm. or less per day, the solution covering it was replaced by fresh solution. Some roots showed no response to this treatment, others increased their rate of growth after the replace-

ment of the solution, and after passing through a maximum, again showed a decline. In some cases a second replacement caused a new burst of growth. In fact one root after a second supply of fresh solution showed a growth of 2.2 cm. per 24 hours between the fifty-seventh and sixtieth day of cultivation, when the experiment was discontinued. These results were interpreted to mean that some material in the grain extract was exhausted by the growth of the root and supplied in the fresh solution. Staling products were not thought important because in some cases similar results were secured when the extract was added to the old solutions.

In a more elaborate experiment, excised corn root tips originally 2 mm. long were grown in 30 cc. of solution in 500 cc. Erlenmeyer flasks at room temperature in the dark. The following media were used: solution C, solution C plus the extract of 0.1 grain per flask, solution C plus 666 p.p.m. of autolyzed yeast, and solution C plus root extract equivalent to five 3 mm. portions per flask. The original root tips were pretreated on agar, and whenever the tips became translucent during their growth the flask was tilted and the root exposed to the air until the translucent condition disappeared, or until danger of injury from desiccation made it advisable again to submerge the tip. By marking the position of the end of the root on the outside of the flask at intervals the rate of growth of each root was noted, and when the rate decreased to 0.5 cm. or less per day the old solution was replaced by fresh. This procedure was continued until the root ceased growth.

A summary of the results of this experiment given in table 6 shows that each of the three extracts was beneficial to the roots. Under the conditions of this experiment the autolyzed yeast was most beneficial, the root extract least beneficial.

The response to the change of medium was very slight in solution C. Only four roots out of fourteen could be interpreted as being favorably affected by a change of solution C, and of these only one showed clear evidence of an increase in the rate of growth, and the effect in this case was not great.

A change in the medium was frequently followed by a marked increase in the rate of growth when solution C plus autolyzed yeast, grain extract, or root extract was used. All of the roots (17) in the

yeast series responded by increased growth rates to the first change of solution, ten of fifteen in the grain extract series, and five of twelve in the root extract series. The number of changes to which a response was made varied with the individual root; some responding to one change only, others to several. The difference between solutions and between individual roots is shown by the average number of days the roots grew in each solution; for solution C it was 13 days (range from 5 to 26); in the root extract, 20 (range from 9 to 36); in the grain extract, 29 (range from 11 to 56); in autolyzed yeast extract, 36 (range from 17 to 100).

TABLE 6

GROWTH OF 2 MM. EXCISED CORN ROOT TIPS IN SOLUTION C AND SAME SOLUTION PLUS VARIOUS SUPPLEMENTS; OLD SOLUTIONS REPLACED BY FRESH WHENEVER RATE OF GROWTH SHOWED MARKED DECLINE. GROWN AT ROOM TEMPERATURE IN DARK

ADDITION TO SOLUTION C	NO. ROOTS	AVERAGE FINAL LENGTH (CM.)	AVERAGE NO. SEC- ONDARY ROOTS	MAXI- MUM LENGTH (CM.)	MAXI- MUM NO. SECOND- ARY ROOTS	MINI- MUM LENGTH (CM.)	MINI- MUM NO. SECOND- ARY ROOTS
None.....	14	9.4	49	22.5	169	2.7	0
Grain extract.....	15	21.2	82	82.0	271	5.8	18
Autolyzed yeast.....	19	41.8	163	145.0	285	8.2	27
Root extract.....	13	19.3	88	53.0	179	5.0	20

The best single example of the beneficial effect of changing the medium was a root which grew in solution C plus autolyzed yeast for 100 days, reached a final length of 145 cm., and produced 285 secondary roots. Its final appearance is shown in figure 2. The rate of growth in mm. per day is shown in figure 3. The solution on this root was changed at the end of 42, 66, 85, and 91 days. In the first period the rate of growth attained a maximum and then declined. The drop in the curve at 11 to 15 days represents a lag caused by the partial drying of the root tip which resulted from tilting the flask in an attempt to correct a slightly abnormal condition of the tip. Replacement of the solution caused a new burst of growth which was repeated in the third, fourth, and fifth periods. As may be noted in figure 3, however, the length of the periods in which growth equaled



FIG. 2.—Root grown in solution C plus 666 p.p.m. of autolyzed yeast, old solutions being replaced with fresh when rate of growth showed considerable decline. Entire root is shown in the three pieces. Note groups of long branch roots at 1, 2, 3, and 4, which are located at positions corresponding to length of root when solution was changed. Original length 2 mm.; final length 1450 mm. Period of growth 100 days. Scale 10 cm.

or exceeded 0.5 cm. per day decreased with each change of the solution, and the maximum rate of growth was also less. In other words, changing the solution became less effective as time went on.

Groups of long branch roots were produced at positions corresponding to the length of the root when the solution was changed. Whether this was a response to the slower growth of the main root tip previous to the change or to the fresh solution is uncertain. We are inclined to think it was due to the latter.

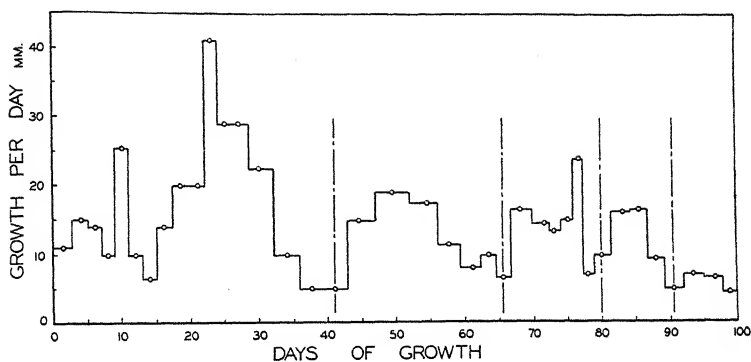


FIG. 3.—Rate of growth in mm. per day of root in solution C plus autolyzed yeast. Broken vertical lines show when old solution was replaced by fresh (see fig. 2).

### Discussion

The problem with which we are primarily concerned may be stated briefly as follows: What are the conditions necessary for the continued growth of excised corn root tips? The media used, composed of solutions of inorganic salts and dextrose, have not proved satisfactory, either because they are deficient qualitatively or quantitatively in one or more nutritional requirements, or because they are injurious on account of a lack of balance, the presence of some specific toxic constituent, or some other condition. While recognizing the difficulty of separating these causes and the possibility that our results may be due to one or to several, we have been inclined to interpret them as due to one or more deficiencies in the mineral nutrient solutions containing dextrose which we have used.



On this basis, the beneficial effects on the growth of excised corn root tips reported in this paper or earlier (3, 7) from the addition to such a medium as solution C of agar, water extracts of agar, qualitative filter paper, water extracts of qualitative filter paper, autolyzed yeast, peptone, corn grain extracts, and corn root extracts would be due to the correction in whole or in part of nutrient deficiencies in that solution. It is possible that a medium, such as solution C, is deficient for the growth of excised corn root tips in two respects; one inorganic in character, which is supplied by the salts contained in agar (or in qualitative filter paper) and the other, perhaps organic, which is supplied in whole or in part by autolyzed yeast, corn grain extracts, and similar supplements. The evidence at hand indicates that the addition of corn grain extract, corn root extract, or autolyzed yeast to solution C or to a modified Pfeffer's solution does not make those media entirely satisfactory for the growth of excised corn root tips. At the same time the beneficial effects of the addition of corn grain extract to solution C plus agar suggests that the latter medium is not entirely adequate.

It is possible that an inorganic medium plus dextrose may be found which is sufficient for the continued growth of excised corn root tips. At present we are inclined to believe that organic material in addition to dextrose is necessary, perhaps organic nitrogen. As a nutrient medium, one containing extracts of agar or qualitative filter paper and dried brewers' yeast, corn grain extract, or peptone in addition to dextrose and to the salts in solution C would seem most promising.

SÖHNGEN (8) found the addition of filter paper to Beijerinck's solution to improve the growth of *Azotobacter* and to increase its nitrogen fixation as much as five times. This was especially true when the filter paper extended above the medium. He explains the results on the basis of adsorption and better aeration.

ALLYN and BALDWIN (1) found the addition of powdered agar, yeast water, and ground filter paper beneficial to the growth of *Rh. trifolii* in certain mannitol media. They are inclined to explain the beneficial effects of these supplements as due to their action on the oxidation-reduction potential of the medium.

We would explain the beneficial action upon the growth of excised corn root tips of the various supplements we have used on the basis of nutrient deficiencies.

### Summary

1. Milk from immature corn grains was injurious or without benefit to the growth of excised corn root tips.
2. The diffusate from germinated corn grains benefited the growth of excised corn root tips in an agar medium containing mineral salts and dextrose.
3. The diffusate into agar from coleoptile tips slightly inhibited the growth of excised root tips less than 1 mm. long; that from root tips slightly favored the growth.
4. Water extracts of the terminal portions of seedling corn roots favored the growth of excised corn root tips in an agar medium containing dextrose.
5. Extracts of germinated corn grains at higher concentrations injured excised corn root tips, producing hypertrophy of the cortex cells. At lower concentrations the extracts were beneficial.
6. Replacing the nutrient solution by fresh solution when the growth rate of excised corn root tips had decreased resulted in little or no benefit when a mineral nutrient solution containing dextrose was used, but caused renewed growth when the solution contained root extract, corn grain extract, or autolyzed yeast.
7. The significance of the experiments from the standpoint of a medium suitable for the continued growth of excised corn root tips is discussed.

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## SECONDARY GROWTH BY MEANS OF A THICKENING RING IN CERTAIN MONOCOTYLEDONS

VERNON I. CHEADLE

(WITH PLATES II, III)

### Introduction

It has long been known that in the arborescent and some herbaceous Liliiflorae, as well as in a number of other groups of monocotyledons, there is an area of more or less radially placed secondary fibrovascular bundles imbedded in similarly oriented parenchyma, both arising from a cambial layer or zone. As a number of years have passed since any work of wide range has been done on these forms, it seems worth while to review, and in some degree to extend, the investigations which have been reported in the past.

The term "thickening ring" is used by SCOTT and BREBNER (24) as the English translation of the German "Verdickungsringe" and of the French "anneau de l'accroissement." There is considerable confusion in the literature concerning the actual connotations of these words; but there seems to be no doubt that the term as here used is at least a part of the full meaning of the word or words. A number of synonymous terms are used in this report, and are not to be confused with the "intrafascicular" cambial activity, which is more widely found in the monocotyledons.

In the course of the research, living plants of the following species were available from the Botanic Garden, Harvard University: Liliaceae—*Aloe saponaria* Haw., *Dasyllirion quadrangulatum* S. Wats., *Sansevieria zeylanica* Willd., *Veratrum viride* Ait., and *Yucca glauca* ((Nutt.) in Fras. Cat.); Amaryllidaceae—*Agave americana* L. and *Furcraea pubescens* Tod. Preserved material of the stems of *Cordyline terminalis* Kunth and *Yucca aloifolia* L. was obtained from the Harvard collection of the Liliaceae. In addition to these forms, Professor I. W. BAILEY kindly permitted access to his prepared slides of various members of the Liliaceae: *Aloe arborescens* Mill., *Cordyline indivisa* Steud., *C. terminalis* Kunth, *Dasyllirion serrati-*

*folium* Zucc., *Dracaena fragrans* Ker-Gawl., *D. goldieana* (Hort.), *D. hookeriana* C. Koch, *Nolina recurvata* Lem., *Yucca aloifolia* L., *Y. filamentosa* L., and *Xanthorrhoea arborea* R. Br. The names and authorities of all these plants were checked in the Index Kewensis (including the 1930 Supplement), except where they occurred in GRAY'S manual (7). The other plants mentioned were checked in the same publications.

### Investigation

#### TECHNICAL PROCEDURE

Material from living plants was killed and fixed either in Carnoy's fluid or in formalin-acetic-alcohol, both prepared according to formulae given by CHAMBERLAIN (5). After proper treatment, following the general directions of CHAMBERLAIN, most of the organs were softened in a 50 per cent mixture of commercial hydrofluoric acid and 95 per cent alcohol. The material remained in this mixture for varying periods of time, depending upon its relative hardness. After subsequent washing in running water for 24 hours and then dehydrating, the various parts of the plants were imbedded in celloidin, following the procedure outlined by JEFFREY (12) and WETMORE (26), and finally stored in glycerin-alcohol.

Macerations of fresh or, when necessary, of killed and fixed material were made by the Cross and Bevan method (alternate treatment with hot sodium sulphite and chlorine water), or by hot saturated caustic potash followed by saturated chromic acid solution in water.

#### GENERAL DESCRIPTION OF SECONDARY BODY

The secondary body is composed of the tissues produced by a peripheral layer or layers of meristematic cells. That portion of the body of tissues on the inner side of the meristematic zone is characterized by vascular bundles imbedded in parenchyma, and that developed on the outer side (usually small in extent) of the dividing layer is characterized by parenchyma alone.

The general appearance of the secondary body has been described by a number of writers, who have discussed at length the elements of the xylem, and to some extent those of the phloem. Those treat-

ments which gave some insight into the general structure of the area under consideration usually dealt with single, or at most a few, species. Therefore the available material from eleven genera comprising eighteen species was studied in the attempt to discover what structures in the secondary body are common to all or a part of these species. Of these eighteen species, all but *Veratrum viride* have been at least mentioned in the literature.

Since this appears to be the first report of secondary growth in *V. viride*, the following is a brief description of the secondary body in this plant. The species in question is an herbaceous perennial with a short, thick, upright stem which gives rise to a large number of leaves whose sheaths form a false "stem." A cross section of the stem just below the point where the oldest functioning leaves are attached illustrates the structure of a normal short-stemmed monocotyledon. The large amphivasal bundles in the central area are imbedded in fundamental tissue, which is composed of cells with somewhat thickened walls, exhibiting many pits. The cortex contains the same sort of fundamental parenchyma. Separating these two areas, however, is a zone of radially oriented parenchyma in which are scattered small collateral bundles (fig. 5). This area, present only in the upper part of the stem, comprises a secondary body somewhat similar to that characterizing the better known species to be described later.

The secondary, collateral bundles consist of a few elements of xylem and phloem. Scalariform tracheids make up the xylem, and short sieve tubes and parenchymatous elements compose the phloem. It is unusual to find scalariform tracheids in secondary bundles; nevertheless the radial seriation of cells in this area indicates that true secondary activity is present in *V. viride*. Moreover, LINDINGER (15) cites not only his own observations, but also those of a number of others, in contending that comparable secondary thickening is present in many monocotyledons possessing a structural organization somewhat similar to *V. viride*. Thus, in the writer's observations, seriation in the stem of *Allium obliquum* L. represents secondary activity, although the peripheral bundles are imbedded in scarcely seriated "conjunctive tissue." (This term is used by EAMES and MACDANIELS (6) to describe the secondary parenchyma in

which the secondary bundles are imbedded.) The same situation, although less striking, is found in *Narcissus pseudo-narcissus* L., *Leucojum aestivum* L., *Hyacinthus orientalis* L., and many similar plants. In those species, however, the meristematic activity does not seem nearly so uniform as that found in *V. viride* and *Sansevieria zeylanica*. Only the latter two species are therefore included in the following discussion of the more typical secondary tissues. The presence of this rather anomalous type of secondary stem thickening in many of the bulbs, corms, or short aerial stems of the Liliaceae and the Amaryllidaceae may have some phylogenetic significance within these two families, but that subject will be considered at a future date.

✧ It may be well to note here the means by which secondary bundles and the conjunctive tissue are distinguished from the primary body. RÖSELER (22) used the following diagnostic characters: (1) the paucity of the phloem elements in the secondary bundles, (2) the oval shape of the secondary bundles (fig. 1), (3) the more or less radial placement of these bundles, and (4) the radial orientation of the conjunctive tissue. In addition, I suggest that the short sieve tubes and lack of spiral or annular elements in these secondary bundles are valuable criteria. These characteristics seem to hold very well, except that the outer or peripheral bundles of the leaf traces often lack the annular and spiral elements also. The infrequent presence of two phloem groups in the secondary bundles (13) is not a good character, for these groups appear to arise from intimate anastomoses of these bundles. A combination of these characters, however, is usually sufficient to make identification of the secondary body reasonably easy.

The secondary area and its constituents were studied from a number of viewpoints. As will be indicated in a later section of this paper, the actual ontogeny of the secondary bundles and conjunctive tissue is fundamentally the same in the various plants examined. On the other hand, the structure of the mature secondary body may vary to such an extent that it provides a basis for identifying the species possessing such secondary tissues.

✓ The placement of the inner group or ring of secondary bundles and the relative thickness of the walls in the surrounding conjunctive

tissue may vary considerably from that characteristic of the subsequently developed secondary body. Furthermore, this irregularity itself is of different magnitude in various species, since the inner area may be very compact or very loose in its construction. For example, in *Dracaena fragrans* it is very compact while in *Cordyline indivisa* the contrary is true, and in *C. terminalis* it may be either. About half the species examined have irregular areas (fig. 2) in the inner region of the secondary body and the other half have regular areas (fig. 3); that is, areas which do not vary from subsequently developed secondary tissues. Furthermore, two specimens of *Yucca aloifolia* exhibited two different types of inner ring formation, one being distinctly irregular and the other regular. Thus there is no characteristic of the first formed secondary tissue which is common to all plants possessing this development.

While there is some variation in the types of bundles present, either in the amphivasal or collateral forms, this seems to be a character which holds throughout the genera. For example, all the secondary bundles in the species of *Yucca* are characteristically collateral, and all those in *Dracaena* are amphivasal. The bundles are amphivasal in *Xanthorrhoea arborea*, but the phloem usually is nearer the outer (distal) portion of the bundles. The same is true of *Agave americana*, both in the rhizome and in the aerial stem; while in *Furcraea pubescens*, whose secondary bundles are small and contain few elements, there is a tendency toward variation from a typical amphivasal condition to one in which the tracheids do not quite surround the phloem. *Aloe arborescens*, contrary to PROLLIUS (21), has mostly amphivasal bundles.

There are only minor variations in the xylem parenchyma and in the conjunctive tissue in the species examined. All of the bundles contain at least a few xylem parenchyma cells. The walls of these cells are apparently always lignified; at least they stain with safranin. The position of the xylem parenchyma at the periphery of the bundles (also commonly found adjacent to the phloem) appears to conform more or less to generic lines. *Dracaena* possesses such an arrangement of the xylem parenchyma, as do most of the *Yucca* species, while the *Aloe* species do not. The apparent lack of conformity in *Dasyllirion*, as well as in *Yucca*, may really be due to in-



ability to differentiate between the xylem parenchyma and the juxtaposed conjunctive tissue. In contrast to the cells of the latter, those of the former are usually slightly elongated and less broad in the radial and tangential directions. Also, where the walls in the conjunctive tissue are thickened, wall thickening is completed in the xylem parenchyma first. There appears to be considerable variation in the wall thickness of the secondary parenchyma area of the species investigated, but in general the thickness of the walls in this area is consistent throughout the genera. For example, as RÖSELER (22) commented, *Dracaena* has thick walled conjunctive tissue and *Yucca* has thin walled (except in certain concentric bands which are partly responsible for the formation of growth rings). ✓

The xylem conducting elements are elongate, are often irregular in form, especially in the short stems, and frequently possess branched or forked ends. These conducting elements are surprisingly uniform throughout most of the species studied, with only *Aloe arborescens*, *Furcraea pubescens*, and *Dracaena fragrans* possessing a number of fiber-tracheids in addition to the normal tracheids. In the latter elements, the apertures of the bordered pit pairs are included, lenticular in outline and crossed, while in the former they are extended and more narrow. This is a convenient but arbitrary distinction. It agrees in general with the proposal concerning these elements made by BAILEY (1). Also in fiber-tracheids the borders are much reduced. Figure 9 shows the greatest thickness seen in the walls of fiber-tracheids. In all the elements considered, the pits are usually numerous and present on all walls. Apparently there is a correlation between the number of pits and their shapes. Thus the more numerous they are, the more oval are their apertures. Likewise, as is the common condition among tracheids, the thicker the walls, the narrower are the apertures. Among the well known monocotyledonous plants possessing secondary thickening, therefore, there is little variation in the types of conducting elements in the secondary xylem.

Exceptions exist, however, in *Veratrum viride* and *Sansevieria zeylanica*, for here scalariform tracheids occur in the secondary bundles. This situation may be true in all plants of similar habit which develop secondary tissues. At least in all those examined, similar tracheids were found.

Throughout the species examined, the phloem appears to be rather uniform in its construction and its types of elements; and although no detailed investigation of the phloem was made, most of this area undoubtedly consists of sieve tubes. They are short and have simple transverse sieve plates. Contrary to RÖSELER, the largest elements in the phloem appear to be the sieve tubes. Especially is this true in *D. fragrans*, where, in addition to terminal sieve plates, clusters of sieve fields were distinctly seen on the side walls. Thin walled parenchyma cells, identified by their nuclei, are present in all cases. The small size and position of some of these elements (in *D. fragrans*, for example) justify the conclusion that part of them are companion cells. Like the sieve tubes, neither of these elements elongates to any appreciable extent in the process of maturation. Although there are small variations in the number and size of the elements, there seems to be no obvious method of separating the genera on the basis of the phloem.

The parenchymatous tissue (secondary cortex) cut off to the outside varies in extent, there being but a small amount until the secondary meristem becomes a cambium of true initials, according to SCHOUTE (23). Extreme examples are *Furcraea pubescens* with a comparatively large amount of secondary cortex, and *Dasyllirion serratifolium* with a meager development. The cells are usually rather oblong, with rounded ends in tangential view and rectangular in transverse and radial views. The only appreciable difference in this tissue throughout the species studied is in its extent, and that feature is not characteristic of genera.

Two conclusions may be drawn from the preceding description of the secondary body produced by a thickening ring in the monocotyledons: (1) While secondary areas have in common the presence of distinct vascular bundles separated by secondary parenchyma, variations occur in some features of the structure and arrangement of such tissues; (2) such variations may or may not be typical of a genus or even of a species.

GROWTH RINGS.—LINDINGER (17) discussed the cause of the concentric layering in the secondary tissue in monocotyledonous stems and reviewed the literature concerning this feature of the secondary body. He was able to distinguish three types of such growth layers, calling them annual rings (Jahresringe). CHAMBERLAIN (4), appar-

ently unaware of the work of LINDINGER or of HAUSMANN (8), pointed out growth rings in *Aloe ferox* and *A. pleuridens*. I have found evidence of similar layerings of structure in the secondary body of the stems in a number of plants. This layering in the secondary area is often rather obscure and an explanation of its formation is correspondingly difficult. The boundaries between zones are not distinct, since there are usually no obvious differences in cell sizes or types throughout any single growth ring. Furthermore, the zones may vary considerably in transverse view and may be very irregular in outline. These zones are called "growth rings" in preference to "annual rings," for it is not known whether they correspond to yearly increments of growth.

*Dasyllirion serratifolium*, *Cordyline indivisa*, and *Yucca filamentosa* (given by LINDINGER as a type form) are examples of LINDINGER's second type of ring formation, in which the differentiation of zones depends upon the relative number of bundles in alternating "dense" and "light" zones. In these species the rings are often composed of a single row of bundles. The growth rings in *Dracaena fragrans* are formed as a result of a similar differential frequency of bundles, but they are vague and difficult to identify because of the presence of consistently thick walled conjunctive tissue.

A compact group of bundles imbedded in thick walled conjunctive tissue alternating with more loosely arranged bundles surrounded by thin walled secondary parenchyma explains the concentric layering in *Yucca aloifolia*, *Cordyline terminalis*, and *Dracaena hookeriana*. The bundles are about the same size throughout. A structural pattern of this type is somewhat similar to that found by CHAMBERLAIN (4) in *Aloe ferox* and *A. pleuridens*, except that he described, as the diagnostic characteristics, bundles of a smaller size and parenchyma cells which had only "slightly thicker" walls than those of adjacent zones.

There seem to be, then, several types of such concentric layering in the secondary body. These types appear as a result of differences (1) in the size of bundles, (2) in the relative number of bundles per unit of area, (3) in the wall thickness of parenchyma cells, and (4) in the size and abundance of parenchyma cells. Any combination may occur. However, *Yucca aloifolia*, *Y. filamentosa*, *Cordyline in-*

*divisa*, and *C. terminalis* furnish evidence that any one plan, once established, appears to characterize a species but not a genus.

#### ORIGIN AND DEVELOPMENT OF SECONDARY BODY ✓

The time at which secondary activity begins in the life history of any particular plant which eventually produces a secondary body has been discussed by HAUSMANN (8) and WRIGHT (27). They agree that cambial activity begins in the hypocotyl and then "extends" up the stem. It arises similarly in *Dracaena* and also "extends," although more slowly, down into the roots.

The question of where the secondary meristem (cambium) originates in relation to the apex of the plant after it has passed the seedling stage is of greater interest in the present study. For many years (MILLARDET 20 to CARANO 3) there has been a controversy over this question and the debated points have not yet been fully settled. Opinion has differed as to what constituted the cortex and the stele, and consequently the origin of the cambial zone was assigned by some workers to the pericycle and by others to the inner portion of the cortex. ✓ *orig.*

MANGIN (18) gave the name of cortex to all that tissue which arises from centripetal differentiation, with meristematic origin in the layer just beneath the epidermis, and that of central cylinder to the increment of tissue developed centrifugally. BARANETZKY (2) found various types of development within the stem apex, and stated that the tissues of the cortex and the stele may be composed of cells of various origins. CARANO (3), discussing the origin of the secondary activity, reported that there are no differences in the tissues of the stem which merit the name of cortex or stele until the secondary body is definitely present. SCHOUTE (23), HAUSMANN (8), and LINDINGER (15) produced evidence to show that secondary thickening is merely a result of the continuation of cell divisions in a restricted (peripheral) part of the primary body, that these cell divisions merely become periclinal in nature instead of haphazard, and that there is therefore no real distinction between the primary and the secondary meristematic activity. SCOTT and BREBNER (24), on the other hand, were convinced that the secondary activity set in after the complete cessation of primary differentiation. They used

a vigorously growing stem for their investigations; but many other workers used comparable material in arriving at precisely opposite conclusions.

It is generally accepted that the pericycle is the outer layer of the stele, but if there is no clear differentiation of tissues in the stem tip, as brought out in the preceding discussion, then it seems impossible to locate exactly in which tissue the origin of the thickening ring takes place. Taking into consideration all the observations which have been made, including my own, the general conclusions may be drawn that a large part of the differentiation of primary tissues takes place before there are any secondary tissues laid down by the thickening ring, and that the region occupied either by the inner part of the cortex or by the pericycle gives rise to the cambial zone.

In regard to the type of cambium present, SCHOUTE (23) has shown it to be of an "Etagenmeristem" ("stage" or temporary meristem) form at first, which later is transformed into a true cambium, and he presented considerable detail to prove the validity of his assertions. It may be recalled that SCHOUTE's concept of an "Etagenmeristem" (a definition with which other investigators concur) is that certain cells become meristematic, divide several times and then lose this power, which is subsequently taken over by adjacent cells. MILLARDET (20) found in transverse view a cambial zone consisting of three to four rows of cells; HAUSMANN (8) and LINDINGER (15) agreed with SCHOUTE, and RÖSELER (22) claimed to have found that the cambial initials are strewn throughout the thickening ring. Neither the exact location nor a convincing description of the cambial initials could be found in the literature.

In attempting to describe the shapes of the cambial initials, therefore, one is confronted with the problem of locating them in the cambial zone. The slightly oblique tangential view of the thickening ring or cambial zone of *Cordyline terminalis* (fig. 8) shows that the cells differ from one another in shape. In *Dasylirion quadrangulatum*, however, a similar view (fig. 7) indicates that the cells resemble one another. According to SCHOUTE (23), the meristem becomes a layer of true cambial initials when cells are cut off to the outside; that is, when secondary cortical cells are produced. Consequently the view of *D. quadrangulatum*, at least, should be a picture of the true cam-

bial initial area, for there is a considerable amount of radially oriented secondary parenchyma outside the cambial zone. Hence in describing this area, one is sure to include the actual cambial initials. Reference to the figures shows that the cells in question in the tangential view may be fusiform, rectangular, truncate at one end and tapering at the other, or polygonal. Furthermore all such variations may occur in a relatively narrow region in a single plant (*C. terminalis*). This irregularity is reflected in the mature conjunctive tissue. When the cambial area is relatively homogeneous (*D. quadrangulatum*, *Agave americana*), however, all the cells appear to be more or less equilaterally polygonal. Longitudinal and cross sections of this region in *Aloe arborescens* (figs. 4, 9) indicate that the initials are rectangular in radial view and somewhat irregularly so in transverse. In *Agave americana*, *Yucca glauca*, *Aloe saponaria*, and *Furcraea pubescens* the cambial initials are also variable in form and intermediate in length between those of *D. quadrangulatum* and *C. terminalis*. Tang.  
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Judging from the species I have observed, therefore, there seems to be no justification for the general statement in EAMES and MACDANIELS (6, p. 248) in which the cambial cells are described as rectangular, or for that by STRASBURGER (25) picturing these cells as polygonal.

In general, the cell divisions in *C. terminalis* are in the longitudinal plane, although they may be also somewhat oblique. Those in *D. quadrangulatum* seem to occur in various directions. Nothing of final nature can be concluded concerning the divisions (and the cell contents) in these cells until actual living cambium is used in investigating this phase of the problem. all  
of

The history of the development of the desmogen strands<sup>2</sup> has been recorded by several workers. Reasonably complete observations by the writer for the most part confirm those made by SCOTT and BREBNER (24), HAUSMANN (8), CHAMBERLAIN (4), and others. A single daughter cell, resulting from subsequent divisions of cells originally cut off from the cambial initials, is the center of development of a des. of  
Camb

<sup>2</sup> SCOTT and BREBNER (24) used this term in preference to procambial strand. Since such an incipient bundle is produced by a secondary meristem, their term, originally used by Russow as synonymous with procambial strand, is appropriate.

desmogen strand which eventually matures into a typical secondary bundle. This cell divides in an anticlinal direction to produce two or three rows; then these cells divide periclinally (fig. 12) and finally in haphazard fashion, although still in the longitudinal direction. Ultimately two or three daughter cells, one of which is usually a member of an adjacent radial row, normally enter into the formation of the bundle (24).

In most cases thickening of the walls of the tracheids on the inner (proximal) side of the bundle begins before all divisions are complete, but figure 9 (*Aloe arborescens*) indicates that this normal course of events is not always followed, for here the bundle is practically completed before any of the elements have even slightly thickened walls. Still more pronounced is the lag in thickening in *Yucca filamentosa*.

Many tiers of cells (as seen in longitudinal plane) aid in the production of a single bundle, for, as will be shown later, the conducting elements of the xylem elongate from fifteen to forty times their original length, while the xylem parenchyma and the phloem elements elongate little or not at all. RÖSELER (22) made counts of the mature tracheids in a great number of secondary bundles, and by making comparisons of their initial and mature lengths presented elaborate data to support his contention that only a single cell in any one tier eventually becomes a tracheid. SCOTT and BREBNER pointed out some fallacies in RÖSELER's work, but admitted that not more than a "few" cells in any tier become tracheids. Similar data from my sections of *Dasyllirion serratifolium* and *Agave americana* are comparable with those of SCOTT and BREBNER.

Even though there are but few (not over two or three) desmogen strand initials which become tracheids in any tier, the elements in the bundles at any one level mature in approximately the same order throughout the stem. Also the bundles in any species are generally of the same type, either collateral or amphivasal. The anatomical evidence indicates, therefore, not only that there appears to be a nicely timed mechanism which makes it possible for the bundles involved to differentiate from similar elements following approximately identical steps each time a new bundle is formed, but that whatever influence controls the differentiation of these bundles is active in a whole region of the stem rather than separately in each bundle.

des. of bundle

As the cells in the desmogen strands are being produced through division, all of them become prismatic in form and sliding growth sets in. Longisections of the stem of *Aloe arborescens* (fig. 4), and especially of *Yucca aloifolia*, show that the intertwining of the tracheids makes it impossible to follow their development closely. To illustrate intermediate stages, therefore, and the final result of the elongation of tracheid initials by sliding growth, macerations were made of these bundles from several plants. Table 1 presents the maximum measurements of the mature tracheids.

TABLE 1  
AMOUNT OF SLIDING GROWTH DURING MATURATION OF TRACHEIDS OF SECONDARY BUNDLES; MATURE LENGTHS ARE MAXIMUM MEASUREMENTS

PLANT	INITIAL LENGTH (MM.)	MATURE LENGTH (MM.)	RATIO OF MATURE TO INITIAL LENGTH
Agave americana.....	0.05	1.5	30
Aloe saponaria.....	0.075	1.8	26
Dasyllirion serratifolium.....	0.05	2.0	40
Dracaena fragrans.....	0.05	2.0	40
Yucca glauca.....	0.075	2.5	37

There is some variation in the length of the mature tracheids, but little in the initials. Median measurements of twenty tracheids fall from 0.2 to 0.5 mm. below the maxima given in table 1, and measurements of minimum lengths indicate that fifteen is the smallest ratio. More measurements might well show greater variations.

The macerated material also revealed immature tracheids with thin walls, possessing single nuclei. These isolations, together with the results of counting the elements, give what RÖSELER and SCOTT and BREBNER considered as irrefutable proof that the tracheids arise from single cells and attain their maximum size by sliding growth. Considerable weight is added to that concept by the number of forked and branched tracheids which I isolated from the secondary bundles. Sliding growth also accounts for the connections of the secondary bundles to the old leaf traces, for elongating elements tend to follow along the latter if they happen to develop just below or above them.



## PHYSIOLOGICAL CONTINUITY

MILLARDET, RÖSELER, SCOTT and BREBNER, and others have pointed out connections of the leaf traces to the secondary bundles. It is obvious that, if such bundles are to play an effective part in the transmission of water and nutrient salts from the roots to the leaves, and of elaborated foodstuffs from the leaves to the stems and roots, they must make close connections somewhere with one another and with the leaf traces. It should be kept in mind that physiological connection between the two types of bundles need not take place where the leaf traces quit the stele, for these traces curve inwardly before abruptly entering the cortical region. The union can be attained while the leaf traces are still near or at the periphery of the central cylinder. This point seems to be lost sight of, or perhaps merely taken for granted. Many have commented upon the course of the leaf traces throughout the arborescent stems of species similar to those considered here, and all report that the majority, if not all, of such plants have the same sort of fundamental plan just mentioned. At least the important traces of the leaves are thus arranged. Where the less important lateral traces "pass" into the leaf from the periphery of the stele, it is readily conceivable that there is a still greater possibility for connections to occur between secondary bundles and leaf traces. RÖSELER confirmed MILLARDET's report that there is no secondary activity while the leaf traces still function in their normal manner. On the other hand, SCOTT and BREBNER have shown that the outer, and therefore youngest, leaf traces are in fact completed by the secondary meristem; and according to CARANO, some of the "primary" bundles are actually secondary in their lower extremities.

My observations of the connections of the youngest common bundles to the secondary vascular tracts in *Agave americana*, *Aloe saponaria*, *Dasylirion quadrangulatum*, and *Yucca glauca* confirm the results of SCOTT and BREBNER, although the anatomical situation in these plants is not nearly so clear. Only *D. quadrangulatum* among these forms contained a large amount of secondary tissue.

The anastomosing of secondary bundles (fig. 11) has been noted by every investigator who has reported on this problem, and nothing more needs to be written concerning it except to repeat that the

greatest number of such anastomoses occur in the tangential direction. This would be expected, when the ontogeny of these bundles is recalled.

Moreover, there are undoubted physical connections between the old, non-functioning (from the standpoint of their original attachments) leaf traces and the secondary bundles. Good examples of this were found in all the plants examined, and figure 6 shows that this connection is indeed close and therefore may have some significance. The phloem and the xylem are in intimate contact. In the ultimate expression of this condition, these old leaf traces extend in a horizontal direction throughout most of the secondary body (figs. 1, 3). RÖSELER suggested that old leaf traces perhaps function to some extent as do vascular rays in the dicotyledons and the gymnosperms, for collenchymatous parenchyma (later becoming thick walled) is laid down around them as the cambial zone continues to increase the girth of the stem.

MANGIN (18), LINDINGER (16), and HAUSMANN (8) mentioned observations on the connections of the adventitious roots to the secondary body. In his report on the origin of adventitious roots and their influence on the anatomy of the stems in the monocotyledons, MANGIN concluded that the entire secondary area resulted indirectly from their production, but he was indefinite in regard to their actual origin. The writer has little to offer in the solution of that problem, for early stages in this development were not definitely identified. It seems safe, however, to infer from their positions (fig. 10) that they arise from the same cambial layer which produces the secondary tissues. They are often imbedded within that tissue, and in many sections of favorable material the spreading out of the bundles which connect with the vascular system of the adventitious root is evident. Perhaps this peculiar arrangement (*réseau radicifère* of MANGIN) makes it difficult to determine when a new root is in the process of formation. Such an arrangement has been seen just inside the cambial zone, and it is not beyond reason to interpret such a condition as the preliminary step in the formation of an adventitious root. The production of such roots has a considerable effect upon the appearance of the secondary body. When the roots are large, as in *D. quadrangulatum*, their influence on the secondary body is

correspondingly greater. The anastomosing among the "wing" bundles, as well as among these and the normally oriented secondary bundles, is obvious in longitudinal and in transverse sections of the stem.

From an examination of the anatomical picture afforded by these plants, therefore, it seems that there should be a continuous although at times devious pathway from the adventitious roots through the secondary bundles into the normally functioning leaf traces.

JACOB DE CORDEMOY (11) enumerated the functions of the secondary body. The bundles transport water, nutrient salts, and elaborated foodstuffs; the parenchyma serves as a storage mechanism, and both act as supporting tissues, the function of the latter varying with the number of bundles and the lignification of the elements. HOLM (9), confirming DE CORDEMOY's work, found fatty oil in the rhizomes of *Cohnia flabelliformis*, sugar in *Yucca gloriosa*, and starch in several *Dioscorea* species, all in the secondary parenchyma. It seems safe, then, to infer that the secondary bundles carry elaborated foodstuffs.

To test roughly the physiological continuity within the plant from the standpoint of upward conduction, two whole plants each of *Agave americana*, *Aloe saponaria*, and *Yucca glauca* were carefully removed from their pots, and after being washed in water were placed in beakers of appropriate sizes containing a water solution of eosin. With the exception of two plants, *A. americana* and *Y. glauca*, which were left for forty-eight hours, they remained in this solution for twenty-four hours, with a fan gently playing over them to increase the rate of transpiration. Every two hours, hand sections were made of single leaves to note how far the eosin had traveled.

At the end of the test period, the eosin had reached the leaves in considerable quantity, so the plants were taken out of the solution and hand sections were made of the roots, rhizomes (*Yucca glauca*, *A. americana*), upright stems, and the leaves. As sections of all parts of other specimens of corresponding size had previously been made, it was easy to identify the extent of the secondary tissues. In the plants left in the eosin solution for forty-eight hours, all the secondary bundles and the peripheral group of primary bundles were stained. In the twenty-four hour plants, only the secondary bundles

in the rhizomes and in the aerial stems, together with the upper parts of the leaf traces, were stained. Macerations were then made of the stained portions of the stem, and the elements found checked in identity with the secondary elements which had previously been isolated from untreated plants.

The eosin solution therefore moved through the roots into the outer secondary bundles, then upward to where the secondary bundles unite with the leaf traces (at the beginning of the secondary activity), and thence into the leaf traces. The inward curvature of the latter to the central portion of the stele and their abrupt exodus to the cortex were well shown in this stained material.

These experiments are too few in number, perhaps, to form the basis for any generalization; but it appears that the shortest pathway to the leaves skirts the outer portion of the secondary area, and that consequently the remainder of the secondary bundles are brought into use only in "emergencies." Similar experiments are planned using plants possessing larger secondary bodies, in the hope of ascertaining whether the old leaf traces function as RÖSELER has suggested.

#### SECONDARY GROWTH IN ROOTS

Few critical observations concerning secondary activity in the roots of *Dracaena* were possible in the material available. Nevertheless a short discussion based for the most part on studies of *D. hookeriana* seems pertinent. The roots in this species have the usual generic type of primary structure, with a definite and conspicuous endodermis which stains deeply with safranin. The pericycle in the sections examined is thin walled and either one or two cells in thickness. The isolated strands of vessels and phloem in the pith are similar to those found in *D. fragrans* (fig. 14).

The actual onset of the secondary activity was not seen, but figure 13 indicates a rather early stage in the development. The endodermis is broken, but most of the cells are still in contact. One or two bundles have been produced, together with a small amount of conjunctive tissue. Figure 15 shows a later stage of this activity and there are now two rows of secondary bundles present. The endodermis is discontinuous and several of the cells probably have

been resorbed by the surrounding tissue (14). The radial placement of the bundles in later stages is similar to the condition found in the stem; hence the secondary growth in these roots appears to originate in the pericycle.

In *D. goldieana*, on the other hand, a situation was observed which parallels the descriptions of other species given by LINDINGER (14). The pericycle produced secondary tissues, and following early loss of its meristematic capability became, like most of its products, permanent thick walled tissue. Outside the bulged but unbroken endodermis, radial orientation of cells (indicating meristematic activity) signifies the appearance of a truly secondary cambial zone (14) similar to that in stems.

These observations tend to bear out CARANO's opinion, later substantiated by MANN (19), that LINDINGER was too emphatic in his statement concerning the exclusively cortical origin of the secondary body in the roots of the species of *Dracaena*. It is impossible to interpret figures 13 and 15 in the light of LINDINGER's concept. However, results based on a study of the situation in *D. hookeriana* do not necessarily weaken the generally accepted conclusion that, where there is a large secondary body present, most of the secondary tissue is produced by a generative zone originating in the cortex.

### Summary

1. The structure of the secondary bundles, conjunctive tissue, and secondary cortex of certain monocotyledons is discussed, and the variations or similarities are enumerated. Genera usually may be characterized by the presence of either amphivasal or collateral bundles, and less markedly, by differences in structure of the conjunctive tissue. The elements in both the phloem and xylem and in the secondary cortex are strikingly uniform throughout most of the species examined. The first description of secondary growth in *Veratrum viride* is presented.
2. Growth rings are found in a number of the genera, and depend upon differences in number and position of the secondary bundles, together with variation in wall thickness of the cells in the conjunctive tissue.
3. In the species examined there appears to be no sharp differenti-

ation into areas of distinctly primary or secondary meristematic activity. The cambial initials are of various shapes in tangential view. In the maturation of the secondary bundles, the conducting elements of the xylem attain their maximum length by sliding growth, the present observations confirming the results of certain earlier workers. All other elements of the bundles retain approximately the length of their initials.

4. Early secondary growth in the roots of *Dracaena hookeriana* is due to cell divisions in the pericycle.

5. Intimate structural connections between the adventitious roots and the secondary bundles, and between those bundles and the functioning leaf traces, aid in effecting continuity of the conductive tissues of the stem.

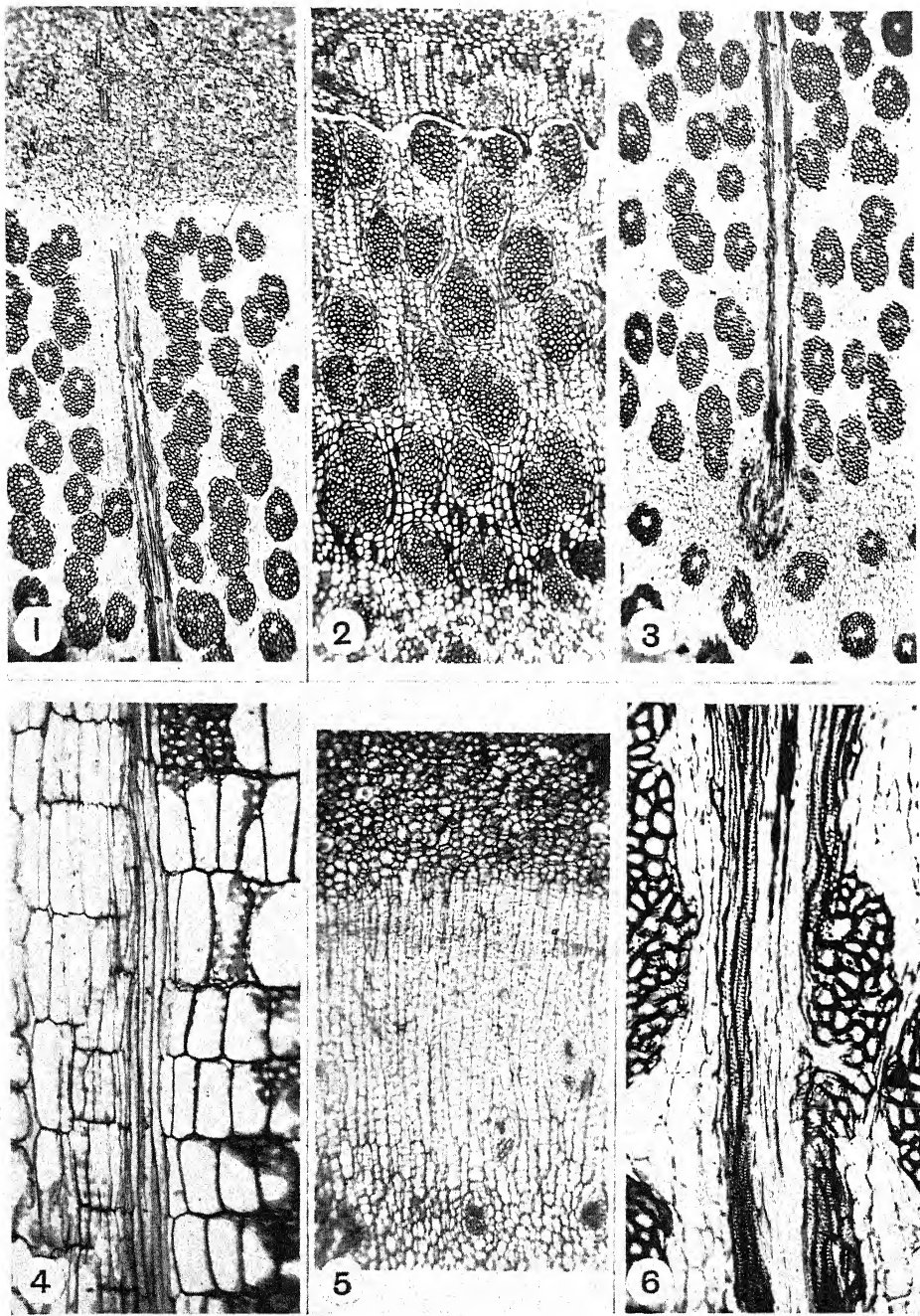
The writer expresses his sincere gratitude to Professor R. H. WOODWORTH of Bennington College and to Professor R. H. WETMORE for helpful suggestions and criticism throughout the course of this investigation.

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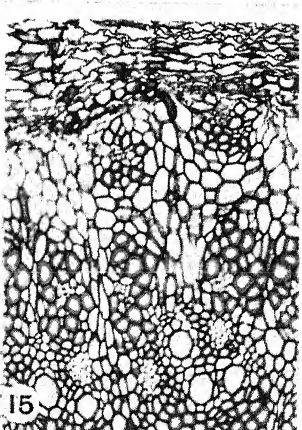
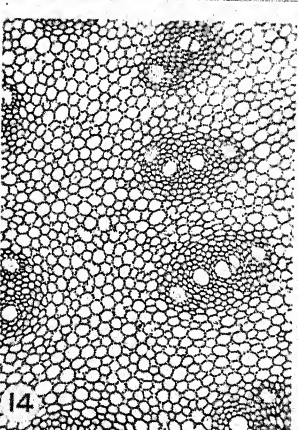
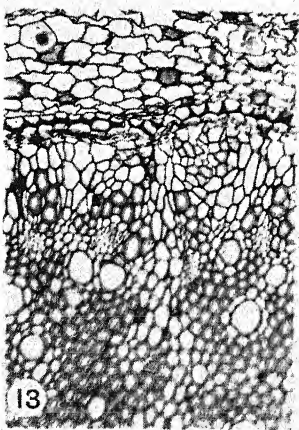
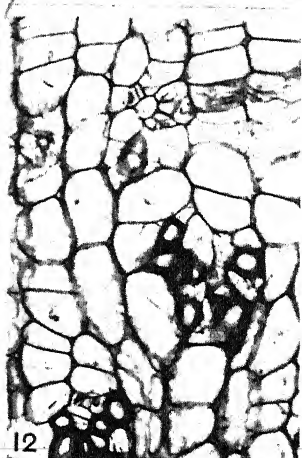
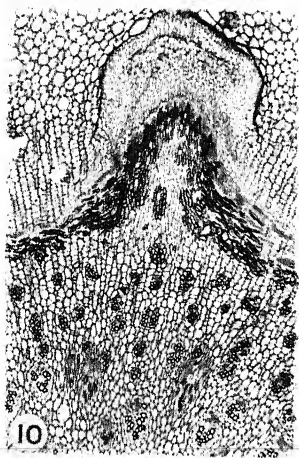
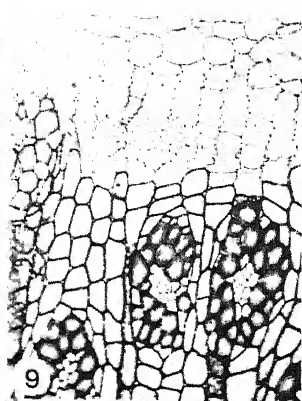
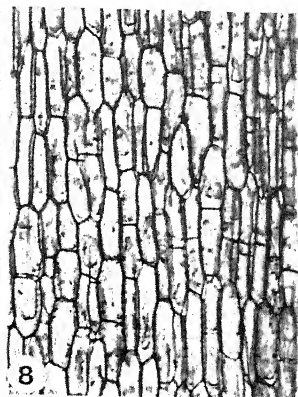
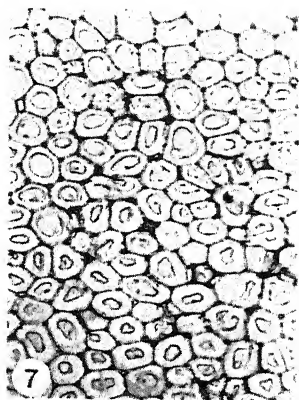
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CHEADLE on SECONDARY GROWTH







CHEADLE on SECONDARY GROWTH



## EXPLANATION OF PLATES II, III

## PLATE II

FIG. 1.—*Dasyllirion serratifolium* Zucc. Cross section of outer part of secondary body and inner part of cortex of stem. Note radial placement of secondary bundles.  $\times 25$ .

FIG. 2.—*Yucca aloifolia* L. Cross section of secondary body of stem. Inner position of secondary body characterized by irregularly placed bundles and thick walled conjunctive tissue.  $\times 30$ .

FIG. 3.—*Dasyllirion serratifolium* Zucc. Cross section of inner part of secondary body and outer part of primary tissues in stele of stem.  $\times 30$ .

FIG. 4.—*Aloe arborescens* Mill. Radial section of stem showing cambial region, immature desmogen strand, and heavily pitted conjunctive tissue.  $\times 110$ .

FIG. 5.—*Veratrum viride* Ait. Cross section through secondary tissues of stem. Note paucity of secondary bundles.  $\times 35$ .

FIG. 6.—*Dasyllirion serratifolium* Zucc. Cross section of secondary body of stem illustrating connection of secondary bundle and leaf trace. Note intimate connection of phloem areas in center.  $\times 110$ .

## PLATE III

FIG. 7.—*Dasyllirion quadrangulatum* Zucc. Slightly oblique tangential section of cambial region.  $\times 90$ .

FIG. 8.—*Cordyline terminalis* Kunth. Section similar to preceding. Cambial initials included in vertical band of cells in center.  $\times 90$ .

FIG. 9.—*Aloe arborescens* Mill. Cross section of stem showing cambial region, immature desmogen strand, and mature secondary tissues.  $\times 85$ .

FIG. 10.—*Furcraea pubescens* Tod. Cross section of stem showing placement of adventitious root. Note secondary tissues of stem on inner side of attachment of root.  $\times 28$ .

FIG. 11.—*Dasyllirion serratifolium* Zucc. Tangential section of secondary body of stem illustrating anastomosing of secondary bundles. Leaf traces in cross sectional view.  $\times 25$ .

FIG. 12.—*Furcraea pubescens* Tod. Cross section of stem showing immature desmogen strand. Fiber-tracheids in mature secondary bundle in lower part of figure.  $\times 200$ .

FIG. 13.—*Dracaena hookeriana* C. Koch. Cross section of root showing secondary tissue under slightly bulged and broken endodermis. Small cells above vessels of primary body of stele indicate inner limits of secondary body.  $\times 80$ .

FIG. 14.—*Dracaena fragrans* Ker-Gawl. Cross section of central portion of primary cylinder of root illustrating "pith bundles" of xylem and phloem surrounded by fibers.  $\times 45$ .

FIG. 15.—Same as fig. 13. Later stage in production of secondary tissues with endodermis now discontinuous.  $\times 85$ .

# MORPHOLOGICAL AND CYTOLOGICAL STUDIES OF *CALLA PALUSTRIS*

MARGARET G. DUDLEY

(WITH PLATES IV, V, AND TEXT FIGURES)

## Introduction

Although *Calla palustris* L., the wild calla lily or water arum, has received considerable attention from systematists, and has been of interest to cytologists, certain morphological and cytological features have not yet been accorded adequate treatment.

BUCHENAU (3) reported CASPARY as citing 272 cases of *Calla* as possessing two, three, or even four spathes on a single spadix, a fact noted also by HALSTED in 1874, and verified in connection with the preparation of this paper. KOSCHEWNIKOFF (12), writing on the development of the flowers of the Araceae, included a description of the inflorescence of *Calla palustris*. His statements have been verified, except those concerning a second type of placenta found in the lower flowers only.

ENGLER (5) monographed the Aroideae and, in a short paragraph on *Calla palustris* (6), stated that it is proterogynous, that the stamens dehisce sporadically after the stigmas have nearly all withered, and that the plant has therefore to rely principally on cross pollination (by means of insects). Later ENGLER and PRANTL (7) classified *C. palustris* as a close relative of *Lysichiton*, *Symplocarpus*, and *Orontium*. The validity of this arrangement will be discussed in a subsequent section. KRAUSE (13) repeated the substance of LIERAU's (14) description of the root of *Calla*, amplifying it somewhat.

ARBER (1) traced (in the Araceae) the "course of differentiation from the family type to the specific type," and made several scattered references to *C. palustris*, with a number of figures illustrating the vascular bundles, the circles of adventitious roots, the ligular leaf sheath, and the spathe. METOLITZKY (16) described and figured the seed of *C. palustris*.

JÜSSEN (11) discussed the haploid generation of the Araceae, devoting considerable space to *C. palustris* and to *Symplocarpus*, but unfortunately omitted *Orontium* and *Lysichiton*, the other two genera of the Calloideae. The only point in which my observations do not agree with those of JÜSSEN is that the microspores show, in a number of cases, division of the generative nucleus while within the anther; they are therefore not binucleate as JÜSSEN has described them.

MARIE-VICTORIN (15) gave an account of *Calla palustris*, with a sketch of the flowering shoot, in which there are several errors: The filaments are much too slender, at least for the species as it occurs in Minnesota. The leaf venation is not correctly drawn, thus giving the impression that the "midrib" persists to the apex of the leaf. The inflorescence arises laterally, instead of being terminal, and the spathe has the appearance of encircling the spadix, which it does not do. The second leaf has no sheath, so that the peduncle apparently arises from the axil of the first leaf.

ERTL (8) discussed the development of leaf venation of the Araceae, including that of *Calla*, but the information contained in his publication has not been checked by the writer.

#### Material and methods

All the material was obtained in the state of Minnesota, from Itasca State Park, Cedar Bog in northern Anoka County, and from Grand Marais on Lake Superior, and consisted of spadices, rhizomes, and mature seeds. The spadices were fixed as soon as possible in either Navashin's or Carnoy's fluid, the latter solution being used for the stages which were young enough to admit of the possibility of finding reduction division in the pollen mother cells. The rhizomes, collected in late October, were covered with damp sphagnum and left in the open until thoroughly frozen (two-six weeks). They were then thawed gradually, planted in peat, and transferred to the greenhouse, where they began to grow almost immediately, blooming six weeks later, after producing three or four leaves. Some of the fruits, gathered in August, were placed in damp sphagnum, where the pericarps and mucilage disintegrated, setting free the seeds, which were then thoroughly dried before planting. Others were allowed to dry *in situ* and stored until needed.

All the material, after being dehydrated, was transferred to chloroform and imbedded in paraffin. The celloidin method was employed in the preparation of the rhizomes, because of their spongy nature. The seeds were soaked in weak hydrofluoric acid for three or four weeks, then imbedded according to the butyl alcohol method.

The sections of the root tips were cut 6–7  $\mu$ ; those of the spadices 12  $\mu$ ; those of the seed, seedling, and mature embryo 15  $\mu$ ; and those of the rhizome 20–30  $\mu$ . Some of the rhizomes were stained with Delafield's haematoxylin, but safranin and fast green were used for the majority of them, and also for the seeds, seedlings, and growing points. Heidenhain's iron-alum haematoxylin was used for the embryos, crystal violet for the root tip sections, and iron-alum haematoxylin and crystal violet for the pollen mother cells.

### Investigation

#### GENERAL DESCRIPTION

*Calla palustris* is found in bogs and swamps of the northern hemisphere (13), occurring locally in moderate abundance in Newfoundland, eastern Canada, the Maritimes, Manitoba, and northern Saskatchewan, and sporadically in Alberta and British Columbia (only one record from British Columbia<sup>1</sup>). In the United States it is found as far west as Minnesota and as far south as Virginia. It is fairly abundant in central and northern Minnesota, except in the western portions, and blooms freely when not too shaded. The plant spreads over the mud by means of a branching sympodial rhizome, green in color, bearing circles of adventitious roots at the nodes. A bud is formed at each node, but very few of these develop into shoots. The leaves, which are confined to the current year's growth (the last few nodes), are, like the buds, alternate in arrangement. The inflorescence, as in other Aroids, is borne on the end of a terminal two-leaved shoot. A branch shoot arises in the axil of the penultimate leaf of this flowering shoot, and grows rapidly, producing a number of leaves (six–twelve normally) the first season. After remaining dormant during the winter, this rhizome bears two more leaves and an inflorescence, which terminates its growth. The plant

<sup>1</sup> HENRY J. K., Flora of Southern British Columbia and Vancouver Island.

axis is then continued by another shoot, arising, like its predecessor, in the axil of the penultimate leaf of the flowering shoot (fig. 1). The rhizome, since it is usually submerged, is very spongy. Its large lacunae are filled with mucilage, and often contain raphide cells, which are much larger than those composing the lacunar walls. The cells containing the raphides are usually attached by one end to the small ones bordering the lacunae, and project therefrom into the mucilage-filled canals (fig. 8). The walls of the lacunae are one cell in thickness. In cross section they are dotted with scattered amphi-

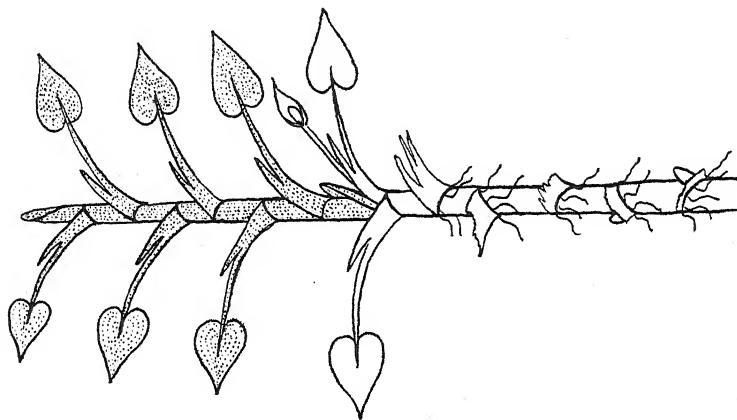


FIG. 1.—Diagrammatic representation of sympodium of plant axis; continuation shoot stippled.

vasal vascular bundles, which frequently appear to be composed of two or three smaller bundles grouped together. The tracheae are few and very weakly lignified. Scattered laticiferous ducts, in connection with the vascular bundles, are evident in stained preparations (figs. 3, 8). Some of these vascular bundles form a ring at each node, outside which, in the young shoot, may be found a circle of adventitious root initials (fig. 9).

The adventitious roots are polyarch, with about seven small protoxylem vessels alternating with the sieve tubes near the periphery of the stele, and an approximately equal number of large metaxylem elements surrounding the small pith. The inner portion of the cortex is composed of radiating rows of regular, more or less



octagonal cells separated by large intercellular spaces, which are lacking between the smaller cells of the outer four or five layers.

The large, thin, cordate leaves have long spongy petioles concave on the adaxial surface. They have a divergence of one-half, and their vernation is convolute, successive leaves being rolled alternately right and left. The numerous well marked lateral veins diverge, one at a time, from the "midrib" and curve outward, parallel to one another, anastomosing just before reaching the margin of the leaf. In consequence the "midrib" gradually becomes narrower and finally disappears, its total length being about two-thirds that of the leaf blade. Each leaf possesses a ligular sheath (convolute in the bud), united with the petiole for approximately half its length and encircling the younger leaves (figs. 2, 4). The line of divergence from the rhizome being in the form of a spiral, with the ends overlapping, the nodes are in consequence not quite at right angles to the long axis of the rhizome, but are slightly slanting, the higher side of the node being alternately right and left (fig. 1).

The inflorescence appears in May (in Minnesota), when the plant has three or four leaves, two on the flowering shoot and one or two on the branch which is to continue the growth of the rhizome. As a rule there is not more than one inflorescence per year from each growing point, but there may be more than one spathe to a spadix (3).

#### MORPHOLOGY AND DEVELOPMENT OF POLLEN GRAINS

The anthers, which are very short in proportion to the filaments, are four-chambered and open extrorsely. The anther wall consists of three layers of cells: an epidermal, a palisade, and a tapetal layer (fig. 10), the first two of which are entirely normal in appearance and behavior.

The pollen mother cells undergo meiotic division while the archesporial cell is present in the ovule. They are large, easily stainable, and possess a conspicuous nucleolus. Previous to division the chromatin material of each nucleus becomes aggregated into a small, deeply staining sphere in close proximity to the nucleolus and very similar to it in appearance (11).

The meiotic divisions (figs. 11, 15) of the successive type take

place rapidly, and result in a considerable number of ellipsoidal pollen grains, so crowded as to be almost indistinguishable. These pollen grains are easily stainable throughout their development up to the point when they finally become separated from one another. They then apparently increase in size, without any appreciable increase of their plasma content, until the nucleus undergoes division. The pollen grain, while still uninucleate, possesses a large central vacuole, and its nucleus is often found near one end, imbedded in the peripheral layer of cytoplasm. Mitosis apparently takes place rapidly in this nucleus, since it was observed but rarely. The nuclei resulting therefrom, although at first identical, later become unequal in size, and are arranged in a line along the longitudinal axis of the pollen grain. Although JÜSSEN states that in *Calla*, division of the generative nucleus does not take place while the pollen grain is still in the anther, 2.5 per cent of the pollen examined by me was discovered to be trinucleate (fig. 16). As these pollen grains were not yet ready to be discharged, the percentage of trinucleate ones would doubtless be much higher at the time of dehiscence.

According to JÜSSEN, *Symplocarpus foetidus* has binucleate pollen, while that of *Zantedeschia aethiopica* and *Z. albomaculata* is trinucleate. In this respect, at least, no close affinity between *Calla* and *Symplocarpus* seems to be indicated.

The haploid chromosome number in *Calla*, as determined from the meiotic divisions (metaphase I, end view, and very late diakinesis) in the pollen mother cells is 18 (figs. 11, 15). The diploid number, as obtained from sections of the root tips, is 36 (fig. 13). Chromosome counts have not yet been recorded for any considerable number of the Araceae, but the basic number in the family appears to be 8 (9), *Spathiphyllum patinii* and *Acorus calamus* (unpublished data) being the only Aroids thus far discovered to have 9 as their haploid number. The data concerning the chromosome number in *Peltandra undulata* have apparently been misinterpreted. The haploid number is given by GAISER (9) as 22, on the authority of DUGGAR (4), but was apparently inferred from his writings to be the haploid number, since it is clearly shown by his illustrations to be the diploid number, if any. DUGGAR (4) states that there are about 22 chromosomes, referring to the first meiotic division, but neglects to state

whether the chromosomes occurred in *Symplocarpus* or *Peltandra*, or in both! The chromosome number for *Zantedeschia aethiopica*, as determined by OVERTON (18), is given as 16, while MICHELL (17) calculated it to be 12. OVERTON worked with greenhouse plants, however, and MICHELL with the native South African ones, which may account for the discrepancy between their results. It is obvious that much additional investigation is needed before any reliable conclusions can be drawn in regard to the basic chromosome number of the family, and as to what extent this criterion can be used as an indication of relationship and phylogeny.

In figure 16, illustrating the trinucleate pollen grain, may also be seen a smaller spherical body, which is one of the nuclei originally belonging to the tapetal layer already mentioned. The cells of this layer soon become dissociated, lose their walls, and form the periplasmodium typical of the Araceae (11), of which the nuclei only are still in evidence by the time the nucleus of the pollen grain is undergoing division. As may be seen from figure 16, the nuclei of the periplasmodium are slightly smaller than those of the pollen grains among which they are scattered, and contain a number of conspicuous chromatin granules, in addition to a nucleolus.

The mature pollen grains, in their natural condition, appear trapezoidal, with two lengthwise contraction grooves. When expanded by lactic acid and slightly stained with aceto-carmin, they are ellipsoidal and show no grooves. The exine is thick, subpunctate, and possesses three pores, one at the end of the grain slightly to one side and two near the other end, equidistant from its middle point and also from the first mentioned pore.

#### SEED AND EMBRYO

The seeds, of which two to fourteen are imbedded in the mucilage of each red berry-like fruit, develop from anatropous ovules<sup>2</sup> which bend outward from a raised circular placenta (fig. 7).

Double ovules sometimes occur, fused throughout their length (fig. 5), which develop into seeds containing normal embryos.

<sup>2</sup> The origin and development of the embryo and ovule have been omitted from this discussion, as a full description of the embryogeny of *Calla palustris* will be included in a later publication. Figures 14 and 19 illustrate typical stages in the development of the embryo.

The mature seed is brown,  $3.7 \times 1.6$  mm. on the average, and pitted in regular horizontal rows. There are a number of dark spongy blotches at the chalazal end, possibly facilitating imbibition of water by the germinating seed (fig. 20). The pits are depressions in the epidermis, underlain by large subepidermal lacunae. The one-layered epidermis is composed of oblong palisade-like cells. The rest of the seed coat (developed from the outer integument) is formed

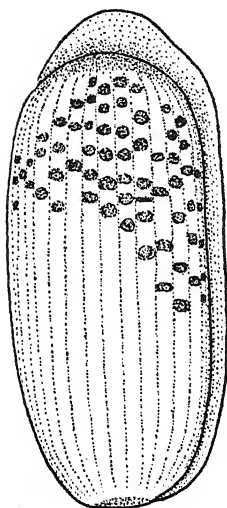


FIG. 20

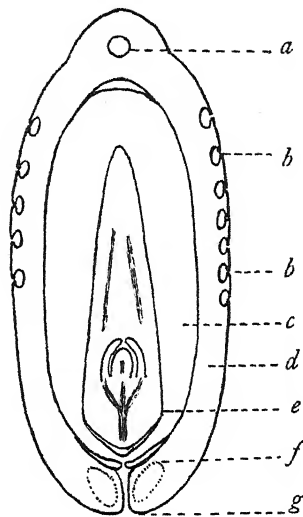


FIG. 21

FIGS. 20, 21.—Fig. 20, external view of seed showing raphe. Fig. 21, diagram of longitudinal section of seed showing embryo (*a*, vascular bundle; *b*, subepidermal lacunae; *c*, endosperm; *d*, seed coats; *e*, embryo; *f*, circum-micropylar canal; *g*, micropyle).

of about twenty rows of small isodiametric cells with intercellular spaces between them. The micropyle is practically closed, and a circular canal is plainly to be seen around it (fig. 21). The raphe possesses a large vascular bundle, and forms a ridge approximately 0.4 mm. wide, extending from the funicle to the chalaza, where it bends over and downward, expanding into a mass of tissue shaped like an inverted funnel. The abundant endosperm, which completely surrounds the embryo except at the micropylar end, is composed of starch-filled polygonal cells having no intercellular spaces. When

the hard testa is removed, the outer layer of the endosperm appears pale yellow and shiny, owing to the adherence of the crushed inner integument.

The mature embryo is green, lanceolate in shape (figs. 17, 18, 21), 2.5 mm. long and 0.6 mm. in its greatest width, central in position at the micropylar end of the seed, and often still attached to the suspensor (fig. 19).

The cotyledon, which forms the major part of the embryo, is thin at the base and sheathes the plumule. At this stage there are three longitudinal veins present which gradually converge, uniting a little below the solid apex of the cotyledon. The lateral veins sometimes unite before coalescing with the midrib, while in other cases they anastomose with the latter independently of one another.

The radicle is short and blunt, with a large central vascular strand, which divides near the apex of the hypocotyl (figs. 17, 21). Branches extend to the cotyledon, and to the leaves of the plumule, of which two are visible at this stage. The first leaf is inserted opposite the cotyledon, is curled inward at the apex over the second leaf, and usually possesses three longitudinal veins, similarly to the cotyledon, although cases were found in which there were four of these veins, two on one side of the midrib and one on the other. The second leaf is essentially similar to the first, and inserted not quite opposite it. The vascular supply to the first adventitious root leaves the main vascular strand immediately below the point where the latter divides into four branches, all of which arise at almost the same level. One of these branches supplies the midrib of the cotyledon, another all the veins of the first leaf, while the remaining two each supply one lateral vein of the cotyledon and part of the veins of the second leaf. Owing to the shortness, almost non-existence, of a hypocotylar region in the embryo, it is extremely difficult to distinguish with certainty the point of origin of the veins.

#### GERMINATION OF SEED

The seeds were planted on damp sphagnum in petri dishes after having been soaked overnight. Those planted on May 5 germinated on May 11. Others planted January 17 (five months after ripening) germinated within four days. Seeds gathered in August germinated

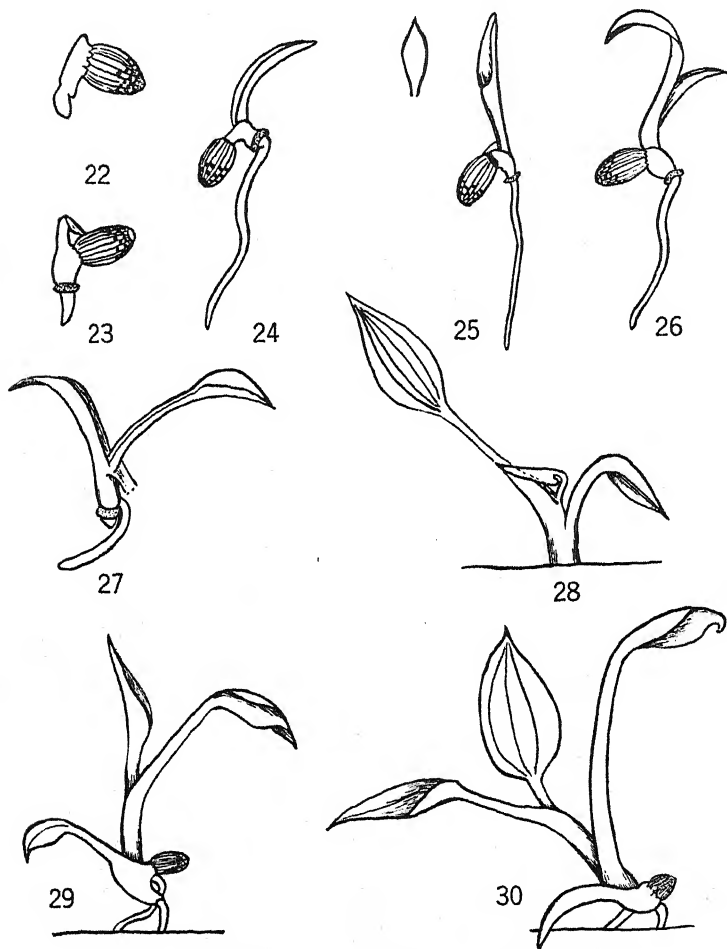
readily in October, after having been thoroughly dried, but would not germinate while still wet.

The radicle, which emerges through the annular canal, is green, massive, and blunt-pointed. It grows rapidly and turns downward, and the plumule is forced out of the seed by the extension of the base of the cotyledon (fig. 23). There is a spongy ringlike protuberance on the radicle, as shown in figures 23-27. The chief elongation of the embryo occurs above this structure, and is evidently due to growth in the basal part of the cotyledon, since in the several sections examined the upper portion still occupied its original position. Germination is evidently according to type B, as defined by BOYD (2), in which germination is hypogeal and there is a shallow collar edging the cotyledonary slit (figs. 24, 25). The three vascular strands of the cotyledon do not traverse this collar, but emerge through the "stalk" of the cotyledon close together while passing down the cotyledonary sheath, then converge to unite with the central vascular strand in the hypocotyl. The vascular supply to each petiole usually consists of three strands, which diverge, as do the cotyledonary ones, in cyclic order from the central mass of vascular tissue.

The leaves of the young seedling are similar in shape to those of the mature plant, but lack the ligular sheath. They have a divergence of one-half, their vernation is convolute, and each has a sheathing base which surrounds the younger leaves. The first leaf, which is usually small and not very well differentiated, emerges from the "collar" previously mentioned with its midrib on the side farther from the seed. Successive leaves are larger and more "*Calla*-like." Unfortunately none of the seedlings used survived beyond the four-leaved stage, so it is impossible to describe their subsequent growth (figs. 22-30).

In about half the cases studied the primary root did not develop at all, but the seedling derived its nourishment through an adventitious root, emerging from the central cylinder at a point immediately below the cotyledonary branches (fig. 12). In external view this root had its origin slightly above the spongy ring of tissue already mentioned, and marking the upper limit of the root sheath. In cases where the primary root grew and functioned, it broke through this sheath and lengthened rapidly. Unlike that of the seedling of

*Zantedeschia aethiopica* (3), the primary root of *Calla* bears no root hairs, but is itself short-lived, its place being taken by adventitious roots. This feature, according to BOYD, is an indication of "ad-



FIGS. 22-30.—Fig. 22, semi-diagrammatic drawing of seedling 2 days old; fig. 23, 4 days old; fig. 24, 7 days old; fig. 25, 8 days old; fig. 26, 11 days old; fig. 27, 13 days old (cotyledon broken off); fig. 28, 21 days old; fig. 29, 20 days old; fig. 30, 33 days old (same plant as no. 11).

vanced morphology" of the seedling; on the other hand, a simple tubular cotyledon, such as that possessed by *Calla*, is to be regarded

as a "primitive characteristic." *Zantedeschia* (*Richardia*) *elliottiana* is the only rhizotomous Aroid discussed by BOYD, and as that is done in a rather sketchy manner, one is not able to make comparisons. The germination of *Zantedeschia* (*Richardia*) *aethiopica*, as described by BUCHENAU, and also as attempted by the writer, seems to be very similar to that of *Calla palustris*, and if carried through might prove to be another link in the chain of evidence binding *Calla* and *Zantedeschia* together.

#### Systematic position of *Calla*

One of the more revolutionary changes in the classification of flowering plants, as set forth by HUTCHINSON (10), concerns the Araceae, and therefore a short résumé of the history of the classification of the Araceae is here given.

BENTHAM and HOOKER (1862-1883) place the Araceae together with the Pandanaceae, Cyclanthaceae, Typhaceae, and Lemnaceae, in series 5 (Nudiflorae), while the Liliaceae are in series 3 (Coronarieae). In ENGLER and PRANTL (1924 edition) the Araceae and Lemnaceae form the 7th "Reihe" (Spathiflorae), while the Liliaceae are placed in the 9th. LOTSY (1911) derives the Araceae, together with the "Spadiciflorae" (Lemnaceae, Cyclanthaceae, Palmae, Pandanaceae, Sparganiaceae, Typhaceae), from the Piperales, and the Liliaceae from the hypothetical "*Pro-ranales*." BESSEY (1915) places the Araceae in the Strobiloideae (ovary superior) and apparently considers them to be derived after the Liliaceae, although this point is not clear. WETTSTEIN (1924) unites the Araceae with the Palmae and the Cyclanthaceae in order 8 (Spadiciflorae), while the Liliaceae are in order 2 (Liliflorae). MEZ (1926) in his sero-diagnostic chart places the allied Araceae and Lemnaceae on the same branch as the Palmae and Cyclanthaceae, but the Liliaceae alone on another branch of the monocotyledons, and further from the main stem. JÜSSEN (1928), after reviewing several systems of classification, agrees with ENGLER in considering the Araceae and Lemnaceae as forming an independent order, and also cites several points of similarity between the haploid generations of the Araceae and the Helobiae (trinucleate pollen grains, periplasmodium, endosperm formation), evidently considering the Liliaceae to be unrelated to either.



HUTCHINSON (1934) places the order Arales (Araceae and Lemnaceae) in the subphylum Corolliferae (with a corolla-like perianth), and derives them directly from the Liliaceae through the tribe Aspidistreae. The Helobiae are all included in the subphylum Calyciferae (perianth biseriate). If this arrangement be correct, the cytological similarities observed by JÜSSEN must be due to parallelism.

HUTCHINSON places *Calla palustris* in the tribe Calleae (number 8 of the series of 17 tribes constituting the family), and it is therefore considered to be more highly evolved than *Acorus*, *Lysichiton*, *Orontium*, and *Symplocarpus* but less so than *Zantedeschia*, *Aglaonema*, *Arum*, and *Arisaema*. According to HUTCHINSON, the more primitive Araceae are those with a poorly developed or leaflike spathe, and hermaphrodite flowers possessing a perianth. He states (10, p. 119), "The more highly evolved Araceae would have unisexual flowers, an increasingly protective spathe, and owing to reduction, some part of the spadix would become barren." An examination of *Calla* readily shows that it is intermediate between these two extremes, and therefore correctly placed with regard to the principles just enunciated.

ENGLER (6, p. 122) likewise places *Calla palustris* in the tribe Calleae, but unites it with the tribe Symplocarpeae (*Lysichiton*, *Symplocarpus*, and *Orontium*) into the subfamily Calloideae, one of whose characteristics is said to be the possession of a creeping underground rhizome [elaborated upon by KRAUSE (13) and which unfortunately, as shown by ROSENDAHL, is not present in either *Symplocarpus* or *Lysichiton* (19)]. Another characteristic stressed by ENGLER is the presence of "simple or unbranched latex ducts in connection with the phloem of the vascular bundles" (19, p. 138). This characteristic, however, is not common to the four genera forming the Calloideae, but occurs in *Calla* and *Orontium* only, thus casting further doubt upon the validity of ENGLER's disposition of these genera.

HUTCHINSON distributed the Calloideae of ENGLER among the three tribes Orontieae (*Lysichiton* and *Orontium*), Dracontieae (*Symplocarpus*, *Dracontium*, *Echidnium*, and *Dracontiodides*), and Calleae (*Calla*), the last being the most advanced and distinguished

from the other two by the absence of a perianth (a characteristic shared by the Monstereae). The Dracontieae are separated from the Orontieae by reason of the possession of a well differentiated spathe (10).

KRAUSE also distinguishes *Calla* from the other three genera (Symplocarpeae) through the absence of a perianth, coupling with this characteristic the presence of endosperm and parallel lateral veins.

A comparison of the four genera seems to show more differences than similarities, and would therefore lead to the conclusion that they cannot be combined into one subfamily, and that HUTCHINSON'S arrangement (in the order of evolutionary progress) is the most satisfactory one yet proposed.

### Summary

1. *Calla palustris* spreads over the ground by means of an alternately branching sympodial rhizome, which bears numerous adventitious roots at the nodes.
2. Each growing point is active for two seasons, producing several leaves the first year and two more the next spring, followed by an inflorescence, which terminates the growth of that branch of the rhizome.
3. Each principal plant axis is continued by the production of a branch in the axis of the penultimate leaf of the flowering rhizome.
4. The leaves are thin, bright green, and cordate, with ligulate sheathes. Their vernation is convolute, and they are rolled to right and left alternately.
5. The ovary is unilocular, and contains from two to fourteen anatropous ovules. Double ovules, fused longitudinally, sometimes occur.
6. A periplasmodium is formed in the anther. The pollen grains are apparently trinucleate before dehiscence occurs.
7. The seed is albuminous, and possesses a hard seed coat. The embryo is central, and germinates without a period of rest if the seed has been thoroughly dried.
8. Germination is hypogeal. The base of the cotyledon elongates, forcing the radicle and plumule down into the substratum. The

cotyledonary slit is edged by a collar, through which the plumule emerges. The vascular strands of the cotyledon are three in number, and do not traverse the collar.

9. The primary root sometimes develops, but more often does not, when its place is taken by an adventitious one. This adventitious root is very noticeable, even before germination.

10. The leaves of the young seedling have no ligular sheath, but resemble adult leaves in all other particulars.

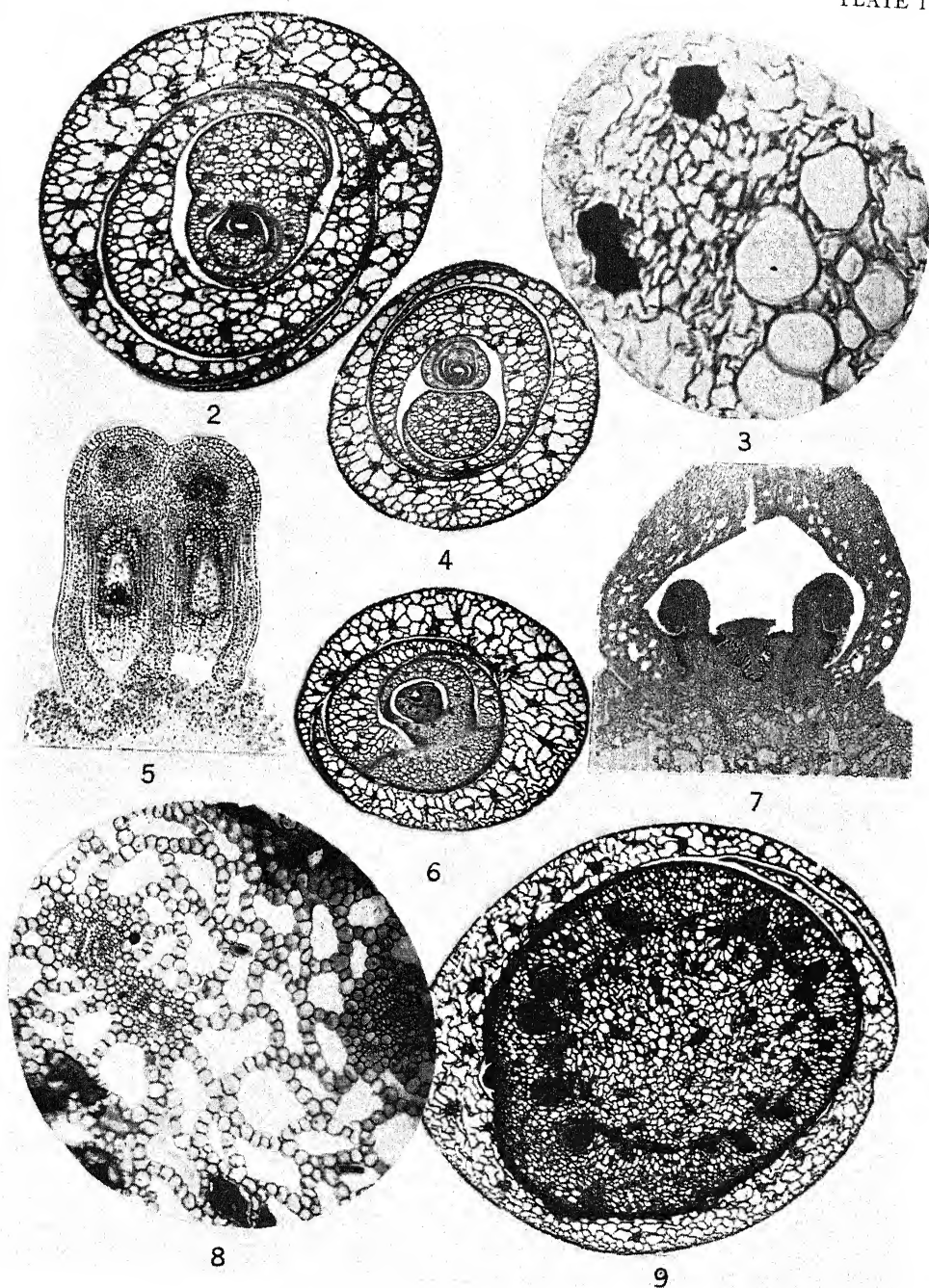
11. The haploid chromosome number, as determined from pollen mother cells at metaphase I, is 18. Metaphase plates of root tips show 36 chromosomes.

The writer wishes to thank Dr. C. O. ROSENDAHL and Dr. F. K. BUTTERS, of the University of Minnesota, for their continued interest and stimulating advice, and Mr. A. O. DAHL for his technical assistance.

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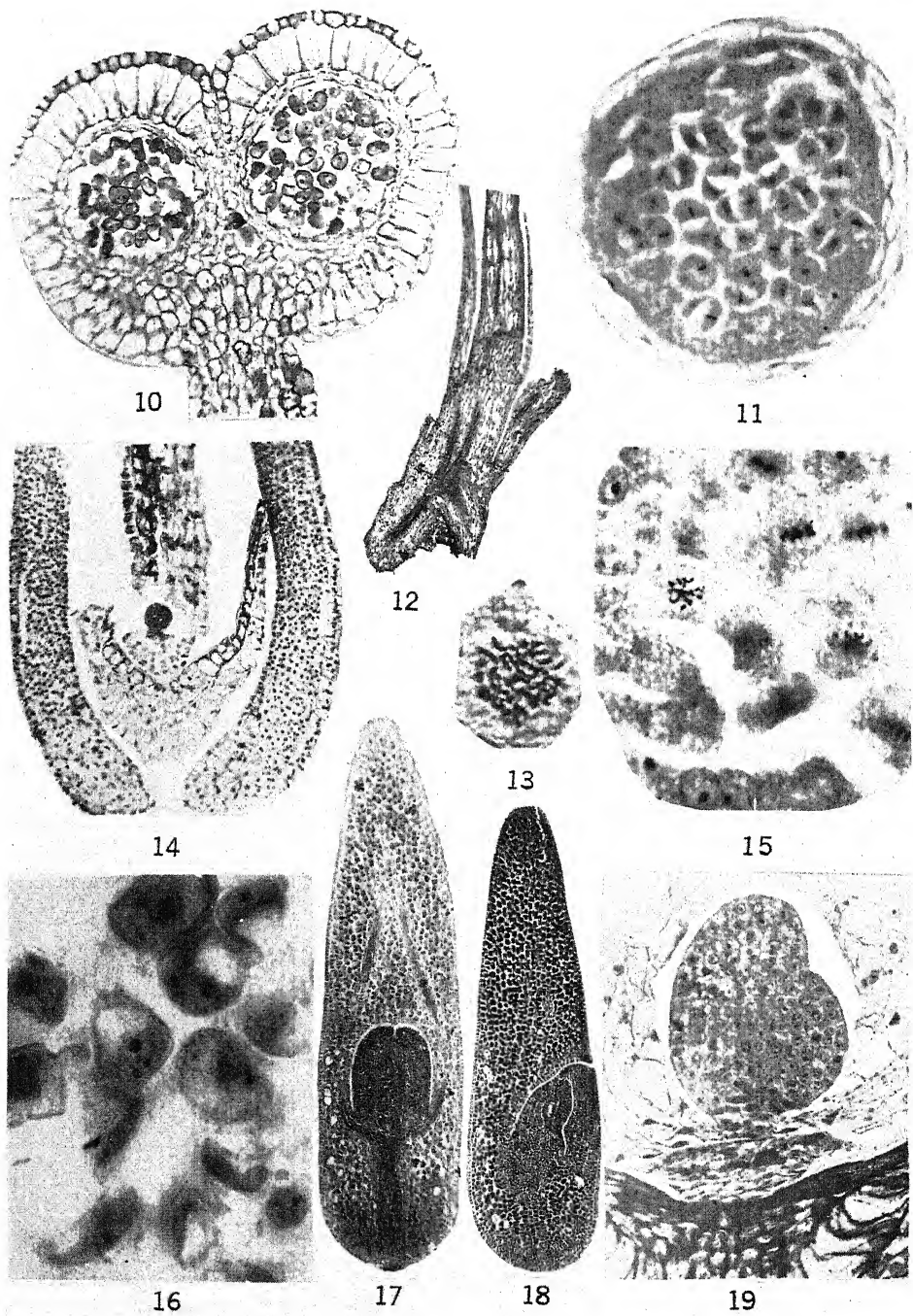
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DUDLEY on *CALLA PALUSTRIS*





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#### EXPLANATION OF PLATES IV, V

##### PLATE IV

- FIG. 2.—Cross section of growing point showing four leaves.  
FIG. 3.—Detail of vascular bundle in rhizome showing laticiferous ducts.  
FIG. 4.—Cross section of growing point, slightly below level at which fig. 13 was taken.  
FIG. 5.—Double ovule, fused longitudinally.  
FIG. 6.—Cross section of growing point showing node.  
FIG. 7.—Longitudinal section of young ovary showing two ovules.  
FIG. 8.—Cross section of rhizome showing vascular bundles, raphides, and mucilage.  
FIG. 9.—Cross section of node showing bud in axil of sheathing leaf.

##### PLATE V

- FIG. 10.—Longitudinal section of stamen.  
FIG. 11.—Cross section of anther sac showing meiotic division in pollen mother cells.  
FIG. 12.—Longitudinal section of 21-day old seedling showing first adventitious root, junction of cotyledon, and vascular supply to cotyledon.  
FIG. 13.—Cell of root tip showing metaphase stage of mitotic division.  
FIG. 14.—Multicellular proembryo showing nucellar cap.  
FIG. 15.—Detail of meiotic division in anther sac.  
FIG. 16.—Mature pollen grains showing one trinucleate grain and one of the nuclei of periplasmodium.  
FIGS. 17, 18.—Longitudinal sections of mature embryos from ripe seeds.  
FIG. 19.—Multicellular proembryo, with suspensor.



# STRUCTURE OF PALEOZOIC SEEDS OF THE TRIGONOCARPALES

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 476

CHARLES L. DEEVERS

(WITH THIRTY-SIX FIGURES)

## Introduction

Much interest has been shown in the seeds of the Paleozoic era since the discovery that many of the fern-like leaves of that period belong to the gymnospermous Cycadofilicales as well as to the true ferns. BRONGNIART grouped these seeds into two classes, the Platyspermae and the Radiospermae. SEWARD classified the 29 genera of these seeds into three orders, the Lagenostomales, the Trigonocarpales, and the Cardiocarpales. The Lagenostomales and Trigonocarpales include BRONGNIART's Radiospermae, and the Cardiocarpales his Platyspermae. These Paleozoic seeds show affinities with the seeds of the cycads.

More than 65 species of *Trigonocarpus* have been described during the past hundred years, mainly as seed casts. The structural details of scarcely more than half a dozen have been given. FIEDLER (3), KRICK (4), and SEWARD (8) have summarized the literature of this group.

Six species of the Trigonocarpales are described in this paper, five of them belonging to the genus *Trigonocarpus*. Following the suggestion of SCOTT and MASLEN (10), the term *Trigonocarpus* has been used rather than *Trigonocarpon*, which was used by WILLIAMSON and NEWBERRY.

The specimens of *Trigonocarpus* studied were collected at Petit Jean Mountain, 12 miles southwest of Morrilton, Arkansas, in Cedar Creek Canyon, about three-fourths of a mile below the waterfall of the same name. They were found literally by the bushel in huge boulders which had fallen down from the eastern side of the cliff. The seeds occur in a hard, gray sandstone in a layer about 3 feet thick which overlies a thick bluish shale. This sandstone layer is 100

feet or so above the bottom of the canyon, and approximately 200 feet below the edge of the cliff. The same stratum appears again on the opposite side of the mountain, where these specimens are numerous. The geologic horizon is upper Atoka, about 150 feet below the Hartshorn sandstone which caps Petit Jean Mountain. The Atoka and the Hartshorn correspond to the Pottsville in age, and therefore represent the Lower Pennsylvanian strata (2). One specimen of the *Trigonocarpales* was collected by Mr. N. J. Gillett in the Strip Mine of the Northern Illinois Coal Company at Wilmington, Illinois. It was found above coal no. 2, which forms the basis of the Allegheny group of the Pennsylvanian system in Illinois.

For detailed studies of internal structure and other anatomical features of these casts, sections of the material were made by means of the diamond saw and carborundum powder. After carefully polishing the faces of the sections, a thin film of canada balsam was spread over them, and they were examined by reflected light. Thin sections of *Trigonocarpus* failed to show any cellular detail, so descriptions are based mainly on surface views. A total of 150 specimens was available, and of these approximately 100 were sectioned. Thin sections and peels were made of the Illinois specimen by the use of the celloidin film method. Histological details were found in these.

Among the material available there are specimens representing at least two previously described species. These are *Trigonocarpus dawesi* Lindley and Hutton and *T. noeggerathi* Brongniart. The same species have been described by several investigators from material obtained both in Europe and America. The remaining species are considered to represent three new species and one new genus and species. None of the specimens of *Trigonocarpus* shows any cellular details, but certain structural features are evident from the regular occurrences of carbonized remains of membranes.

None of the seeds upon which this investigation is based was found directly attached to other plant organs, but several fragments of stems and pith casts of *Cordaitea* were found associated with them. Near the locality where the seeds were found, many casts of *Stigmara ficoides*, some species of *Calamites*, and a few stems of *Lepidodendron* were present. The seeds show no evidence that they were transported or stream worn; they appear to have been fossilized where they fell.

## Description of species

*Trigonocarpus dawesi* Lindley and Hutton (figs. 1-8, 12)

Fifty seeds of this species were investigated, and 35 of them were sectioned. They are of large size, varying in length from 3.5 to 5.5 cm., oval in shape, nearly circular in cross section, and from 1.7 to 2.7 cm. thick. They bear on the surface three nearly equidistant salient ridges or wings. About half of the specimens are winged



FIGS. 1-8.—Fig. 1, *Trigonocarpus dawesi* Brgt., nearly median longitudinal section: *m*, micropylar end of cast; *p*, pollen chamber with top broken and resting on megaspore membrane; *a*, darker layer, the sclerotesta; *b*, carbonized remains of inner flesh; *c*, megaspore membrane-nucellus remains. Figs. 2-4, serial sections from median to outside of same cast. Fig. 5, median longitudinal section of another cast of same species; megaspore membrane-nucellus remains crushed and invaginated. Figs. 6-8, *T. dawesi* Lindley and Hutton: 6, view of basal end showing foramen partly filled with carbonized remains of vascular tissue; 7, side view showing one of three ridges; 8, micropylar end with part of wing still intact at upper right. Note tendency that many specimens have of splitting into three valves.

(fig. 8) and half bear ridges only (figs. 6, 12). These wings and ridges extend the entire length of the seed, from about 5 mm. above the foramen to the micropyle. The wings are approximately 1 mm. thick, and increase in width from near the foramen to a point about 1 cm. from the micropyle, where they are 4 to 5 mm. wide. The ridges are uniform in size.

The sclerotesta is evident in many of the sections as a darker layer, the outermost layer of the cast, and is about 1 mm. in thickness (fig. 1 *a*). It is also evident in the winged specimens (fig. 8) and probably in those having ridges (fig. 12). In many of the specimens the sclerotesta is sharply pointed in the micropylar region; in others it is less pointed or broken. The basal end is rounded, and near its center is a conspicuous foramen (fig. 6).

The inner flesh is represented by a thin carbonized membrane just inside the sclerotesta (fig. 1 *b*). This membrane extends over the entire inner part of the seed from the basal disk to the micropyle, although it is often broken and crushed (fig. 5). In figures 1-4 it is shown in about its original position.

There is no indication of a nucellus separate from the megaspore membrane and the pollen chamber. The latter is evident in many of the specimens as a truncated cavity about 3 mm. high, and in the best specimen is 1 cm. broad at its base (fig. 1 *p*). The membrane inclosing this structure is broken and shrunken, as are the other membranes. The vascular organization is shown poorly, except as a carbonized mass in the foramen at the base of the seed (fig. 6). The nucellus-megaspore membrane is much more prominent than the other membranes; apparently the nucellus had a definite vascular system which is carbonized and included in this complex.

The megaspore membrane appears relatively thick, probably because the nucellar remains are also in contact with it. It is in about its original position in figures 1-4, where it surrounds the cavity previously occupied by the megagametophyte. The megaspore membrane usually is invaginated as seen in figure 5.

The winged specimens represent casts of forma beta as designated by ARBER (1); that is, those which have the sclerotesta as the outermost part. It is possible that the ridged specimens represent forma gamma casts (casts of the inner portion of the sclerotesta only). In the Arkansas material both the ridged and the winged specimens show a foramen at the base, and a darker layer about 1 mm. thick as the outermost layer of the cast. This darker layer is the sclerotesta. It is not known whether the sclerotesta represents the former thickness of this layer, but the sclerotesta of the known *Trigonocarpales* is somewhat less than 1-4 mm. in thickness. Usually the

wings are broken off in removing the seeds from the inclosing matrix, and the seeds have a tendency to split into three nearly equal valves (fig. 8). The question arises whether the specimens possessing wings are to be considered as belonging to the same species as those having ridges, or distinct from them. Those specimens which bear wings along the entire length of the seed closely resemble *Trigonocarpus bertholletiformis* (*T. bertholletiforme* Foster; 5, pl. 42, figs. 12, 12 a); but after allowing for a degree of variation among the specimens available, it seems more probable that they are variants of a single species.

*Trigonocarpus noeggerathi* Brongniart (figs. 9-11, 26)

Fifty seeds of this species were studied, and about 35 were sectioned. They were not so large as those of *Trigonocarpus dawesi*. They vary in length from 2 to 3.4 cm., are ovate, and are nearly circular in cross section. In breadth they have a maximum diameter of 2 to 2.4 cm., and bear on the surface three nearly equidistant wings which extend from near the hilum to the micropyle. These wings are not so prominent as those of *T. dawesi*, and a large number of the seeds tend to break into three equal valves along the line of the wings, in the same manner as the former species.

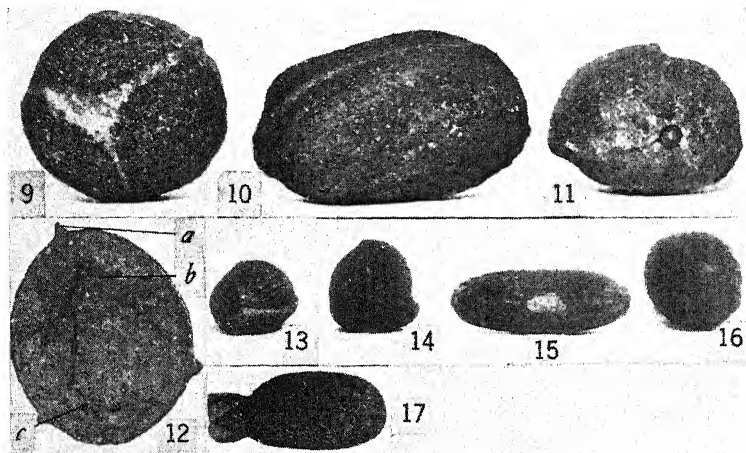
The sarcotesta is represented by a thin carbonized film uniformly thick over the entire seed; and as in the former species there is no indication of the thickness which this part of the integument may have had in the living state.

The sclerotesta is evident, as in *T. dawesi*, as a darker layer about 1 mm. in thickness. It is less pointed in the micropylar region than it is in the former species, and has a blunt appearance (figs. 9, 10). The rounded base has the foramen as has *T. dawesi* (fig. 11).

The inner flesh is represented by a thin carbonized membranous structure immediately inside the sclerotesta (fig. 26 b). This membrane extends over the entire inner part of the seed from the basal disk to the micropyle. Often it is broken and displaced.

There is no indication of the nucellus other than the megaspore membrane and the pollen chamber. Since the innermost membrane of these casts appears much more conspicuous than the membrane representing the inner flesh, it is probable that the nucellus, with its vascular tissue (9), is not separate from the megaspore membrane.

The pollen chamber is evident in many of the specimens as a truncated structure just above the megaspore membrane. The membrane about the pollen chamber is shrunk and collapsed in figure 26, and is not so large, proportionately, as the same structure in *T. dawesi*. The vascular organization is poorly shown, except as a carbonized mass in the foramen. What may have been the extent of the vascular tissue originally present is not indicated, unless the



FIGS. 9-17.—Fig. 9, *Trigonocarpus noeggerathi* Brgt., micropylar end showing three wings, or salient ridges. Fig. 10, side view, micropylar end to left, showing part of two wings. Fig. 11, basal view showing foramen partly filled with carbonized remains of vascular elements. Fig. 12, *T. dawesi* Brgt., cross section at middle of cast: *a*, one of three ridges; *b*, folded and shrunk remains of inner flesh; *c*, megaspore membrane-nucellus complex. Fig. 13, *T. costatus* sp. nov., micropylar view showing three primary ridges with about 20 smaller ones between them. Fig. 14, basal view showing characteristic splitting. Fig. 15, side view, basal end to left. Fig. 16, micropylar view showing slightly pointed apex. Fig. 17, *T. serratus* sp. nov., side view, basal end to right. Three of the six divisions of cuplike structure of summit seen at micropylar end to left.

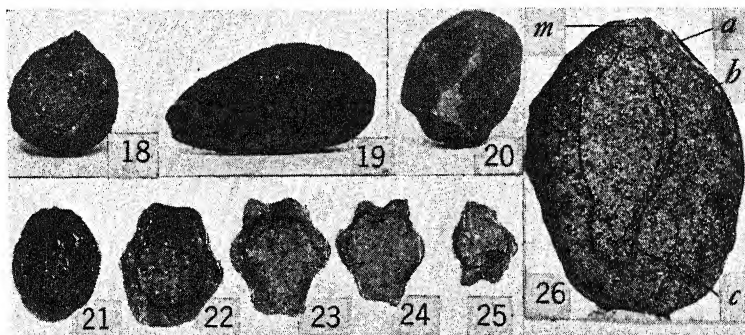
thickness of the layer representing the megaspore membrane-nucellus includes the vascular tissue of the nucellus (10).

These casts vary considerably in size and shape. Again it is possible that more than one species may be represented in this collection, but until better preserved specimens are described it seems best to consider them as belonging to one species.

*Trigonocarpus ouachitensis* sp. nov. (figs. 18-25)

DIAGNOSIS.—A radially symmetrical obovoid seed, flattened at base and circular to hexagonal in cross section. Sclerotesta (length 2 cm., width 1 cm.) produced into a sharp apex, and bearing six ribs (three principal sharp angled and three less prominent). Ridges flatten out equally near the middle of the seed to form an irregular to regular hexagon at the base. Sarcotesta present only in the form of a thin carbonized membrane.

This species is represented by 33 specimens, 15 of which were sectioned. It is much smaller than *Trigonocarpus noeggerathi*, and is



FIGS. 18-26.—Fig. 18, *Trigonocarpus ouachitensis* sp. nov., basal view showing hexagonal form of sclerotesta about base. Fig. 19, side view, micropylar end to left; three of the six ridges apparent. Fig. 20, micropylar end with three major and three minor ribs; apex broken off. Figs. 21-25, serial sections from base to near the micropyle. Fig. 26, *T. noeggerathi* Brgt., near median longitudinal section: *m*, micropylar end; *a*, sclerotesta; *b*, carbonized remains of inner flesh; *c*, megaspore membrane-nucellus, much shrunken.

characterized by the seeds being of comparatively uniform size. While there is little evidence that the three principal ridges were prolonged into a micropylar tube, it is possible that this was the case. The three less prominent ribs end at the micropyle, and in many of the specimens the micropylar end is broken off (fig. 20). There is no evidence in the matrix which surrounded the casts of the presence of a micropylar tube, but it is only after well preserved structural specimens of *T. parkinsoni* were studied that this structure was found in that species (10). The base of the seed is somewhat dome-shaped, resembling in this respect the base of *T. oliveri*

Scott and Maslen (10). It differs from *T. oliveri* in that the base is not so broad, proportionately, and in tapering more sharply toward the micropyle. The base is distinctly hexagonal in most of the specimens. SALISBURY (7) thinks that *T. oliveri* has eight ridges, and that it is not a true *Trigonocarpus*.

There is little structure preserved in these specimens. The carbonized remains on the outside of the casts are apparently much thicker than in the former two species, indicating that the sarcotesta was of much greater thickness in this seed. Figures 21 to 25 show a broken, carbonized, membranous structure, which probably represents the megaspore membrane-nucellus remains. The nucellus was free to the base, and has vascular tissue present.

*Trigonocarpus costatus* sp. nov. (figs. 13-16)

DIAGNOSIS.—A radially symmetrical seed cast, elliptical in longitudinal section and circular in cross section. Sclerotesta (length 10 to 20 mm., width 7 to 10 mm.) slightly pointed at apex, rounded at base, and bearing on the surface three faint, equidistant ridges which extend from the base to the micropyle. Between the ridges occur about 20 faint striae, equally spaced, and running the entire length of the seed (figs. 13, 16).

This specimen resembles *Trigonocarpus multicarinatus* Newberry (5) in that it possesses a number of striae. These ridges are not so prominent as those of the latter species, and the larger ridges are not prolonged into the raised center of a cupped summit as in *T. multicarinatus*. *T. costatus* is slightly pointed at the micropylar end and not truncate as is *T. multicarinatus*.

This species (figs. 13-16) is represented by fourteen specimens, six of which were sectioned. There is considerable variation in size, and many of them were split into three equal valves along the line of the more prominent ridges (figs. 13, 14). Like the other casts, they bear a carbonized mass at the foramen which probably represents the continuation of the inner vascular elements. They are covered with a thin carbonized film representing the sarcotesta. The only other structure present is a membranous mass about 1 mm. inside the surface, which probably represents the megaspore membrane-nucellus complex.



*Trigonocarpus serratus* sp. nov. (fig. 17)

DIAGNOSIS.—A radially symmetrical seed, elliptical in longitudinal section and circular in cross section. Sclerotesta (length 14 mm., width 7 mm.) transformed at apex into a cup-like structure, 3 mm. high, which is divided into six equal, triangular teeth, each 2 mm. long. The body is 11 mm. in length, and bears on the surface indications of three faint, equidistant ridges, between which the surface is smooth. The base is rounded, and has a carbonized mass in the foramen which represents the continuation of the inner vascular elements.

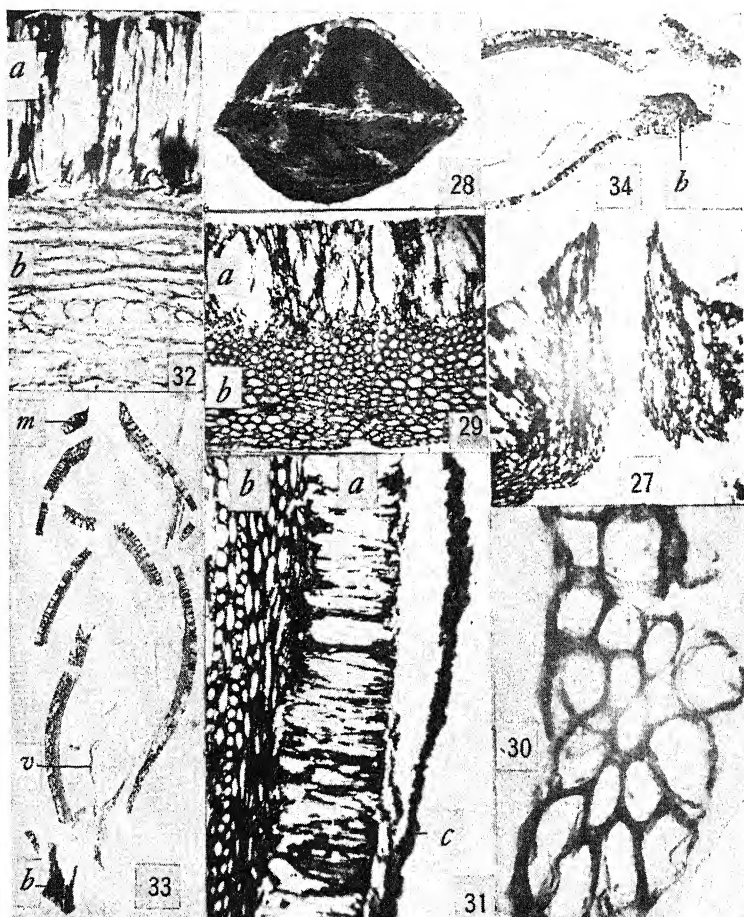
This species is represented by a single specimen, and was not sectioned. It differs from *Trigonocarpus ornatus* Newberry (5) in possessing only three faint ridges where that specimen bears six prominent ones, each of these being prolonged at the micropyle to form a division of the cup-like structure. In *T. serratus* the ridges, although opposite the lobes of the cup, appear to end with the main body of the seed and have no part in the formation of the cup. In *T. ornatus* the ridges are slightly constricted at the neck, and in the specimen studied there is a definite constriction between the cup-like summit and the body of the seed.

*Sarcospermum ovale* gen. et sp. nov. (figs. 27-36)

GENERIC DIAGNOSIS.—A radially symmetrical ovoid seed, circular in cross section, without ribs or sutures. Testa differentiated into three parts, sarcotesta, sclerotesta, and apparently an inner fleshy layer. Nucellus probably free to the base, and shows about 20 vascular strands in cross section.

SPECIFIC DIAGNOSIS.—Seed ovoid, 3 cm. long, with maximum width of 1.5 cm., circular in transverse section. Sarcotesta 0.04 mm. thick in its present crushed condition. Sclerotesta composed of outer palisade layer of thick walled cells 0.5 mm. in extent, and an inner layer (0.5 mm. thick) 13 to 18 elements wide of elongated fibrous cells. Sclerotesta slightly pointed at micropylar end and enlarged at basal end.

There is much similarity in the general plan of structure of the seeds of *Stephanospermum* Brongniart, *Aetheotesta* Brongniart, and *Sarcospermum*. *Sarcospermum ovale* differs from *Stephanospermum*



FIGS. 27-34.—*Sarcospermum ovale* gen. et sp. nov. Fig. 27, near median section of micropyle showing short micropylar tube. Fig. 28, surface view of seed showing part of base covered by inclosing matrix; micropylar end to right. Fig. 29, portion of cross section of seed coat: *a*, palisade cells of outer portion of sclerotesta; *b*, inner layer of sclerotesta, composed of long, isodiametric, fiber-like cells. Fig. 30, cross section of vascular bundle of nucellar vascular tissue, a mesarch bundle composed of scalariform tracheids. Fig. 31, portion of near cross section of sarcotesta and sclerotesta: *b*, fibrous layer; *c*, remains of sarcotesta, split and crushed. Fig. 32, portion of longitudinal section of sclerotesta. Fig. 33, near median longitudinal section showing badly crushed condition of tissues: *m*, micropylar region; *v*, vascular elements of nucellus; *b*, portion of base. Fig. 34, median longitudinal section of base of seed.

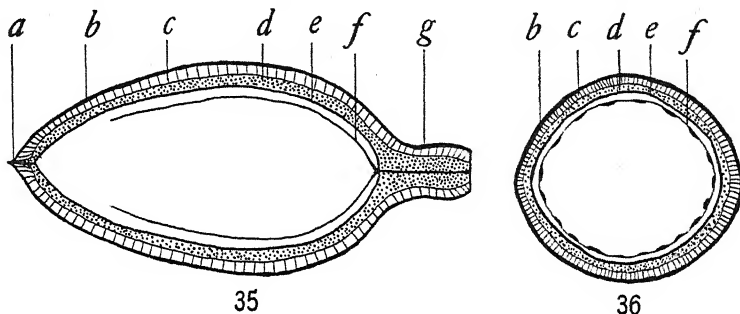
*akenoides* Brongniart in being of a much larger size, in lacking the long micropylar tube surrounded by a prominent crown, in having a much larger base, and in having the nucellar vascular elements divided into definite strands rather than being in a continuous sheath. *Sarcospermum ovale* differs from *Stephanospermum caryoides* Oliver (6) in being about twice the size of that specimen, in lacking the abbreviated crown, which has a hairy epidermis in the micropylar region, and in the structure of the nucellar vascular system as in *Stephanospermum akenoides*. In general structure *Sarcospermum ovale* approaches *Aetheotesta elliptica* Renault somewhat more closely than it does the other two species. *Sarcospermum ovale* differs from this specimen principally in having a much more enlarged base, and in possessing a sarcotesta which is of uniform thickness over the entire seed, rather than one that is much enlarged on both the basal and the micropylar ends and thin about the middle. The micropylar portion of the sarcotesta in *A. elliptica* is lacunose in nature, and the sclerotesta is prolonged into a micropylar tube. The nucellar vascular systems in *A. elliptica* and in *S. ovale* are divided into definite strands, but they appear to reach farther into the seed cavity in the latter.

This seed from Wilmington, Illinois, was so crushed and broken during the process of fossilization that it is difficult to determine accurately its former shape (fig. 33).

The sarcotesta was present over the entire seed, except at the micropylar canal, and is of uniform thickness. It was so crushed and decayed that it is impossible to determine its former thickness. This layer is split in places (fig. 31 c), and parts of it appear outside of a layer of calcite, about 1 mm. thick, which surrounds the seed.

The sclerotesta completely invests the seed except at the upper end where it is perforated by the micropylar canal (fig. 27). This tissue is composed of two distinct layers of cells. The outer is a single row of thick walled palisade-like cells which extend at right angles to the inner layer. These cells are about 0.5 mm. in length, and are of uniform thickness over the seed except at the micropylar and basal ends. In the region of the micropylar beak they are considerably elongated and gradually come to take a position nearly parallel to the vertical axis (figs. 27, 35 a). In the extreme basal

region these palisade cells are much elongated and are at an angle of about  $30^{\circ}$  to the longitudinal axis (figs. 34, 35). They are distinctly separated from the layer beneath, and are mostly filled with a carbonized, opaque substance. These palisade-like cells appear to be the outermost layer of the sclerotesta. The inner layer is composed of elongated thick walled cells which are isodiametric in cross section (figs. 29, 32). These cells extend lengthwise in the seed for the most part, but are often tortuous and twisted irregularly about one another, especially in the basal region (fig. 34). This tissue makes



FIGS. 35, 36.—Fig. 35, reconstructed median longitudinal section of seed: *a*, micro-pyle; *b*, remains of sarcotesta; *c*, palisade layer of sclerotesta; *d*, fibrous layer; *e*, inner flesh; *f*, vascular tissue of nucellus; *g*, large base of seed. Fig. 36, reconstructed cross section of middle of seed: *f*, nucellus showing about 20 vascular bundles.

up the greater part of the relatively large base of the seed, which is about 4 mm. long and 4 mm. thick below the body of the seed (fig. 34). The innermost layer of the sclerotesta is composed of the same elongated thick walled cells as the rest of the fibrous layer, and does not have the appearance of a definite epidermal layer.

There is no definite tissue which may be called the inner flesh, but there is a region just inside the sclerotesta which has a darker color and appears to be the debris from some tissue. This, and the fact that the sclerotesta has no finishing layer, or epidermis, would indicate that the inner flesh was present.

There is some question as to whether this seed shows a nucellus. At some distance inside the sclerotesta appears a definite membranous structure, which is much shrunken, and bears on its inner face about 20 definite vascular bundles. There are traces on the out-

side of this membranous structure of thinner walled tissue which has an amber colored material inside the cell walls. It cannot be determined definitely whether these belong to the integument or to the nucellus, but apparently they belong to the latter structure. The vascular bundles are made up of scalariform tracheids, and each bundle contains from a few of these elements to more than 25 (fig. 30). The bundles appear to be mesarch, with the smallest elements near the center of each bundle although it was not definitely determined whether these were protoxylem. The vascular system extends from the base of the seed cavity to a region in the neighborhood of the probable location of the pollen chamber. There are no vascular elements through the base of the seed, contrary to expectation, nor was a pollen chamber seen. There is no indication of any tissue inside of the nucellar vascular system.

### Summary

1. A histological study was made of about 100 sandstone casts from the Lower Pennsylvanian strata of Arkansas, and one seed coat from Illinois.

2. Six species of the Trigonocarpales are described, five of them belonging to the genus *Trigonocarpus* Brongniart, and one being a new genus and species. The plants described are: *T. dawesi* Lindley and Hutton, *T. noeggerathi* Brongniart, *T. ouachitensis* Deevers, *T. costatus* Deevers, *T. serratus* Deevers, and *Sarcospermum ovale* Deevers.

The writer wishes to acknowledge the assistance received from various members of the Department of Botany of the University of Chicago, where the investigation was conducted.

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# EMBRYO ABORTION IN THE PEACH IN RELATION TO CHEMICAL COMPOSITION AND SEASON OF FRUIT RIPENING<sup>1</sup>

H. B. TUKEY AND F. A. LEE

(WITH TWO FIGURES)

## Introduction

That early-ripening varieties of the peach (*Prunus persica* Batsch.) produce abortive seed has been pointed out by CONNORS (3) and CHANDLER (2). TUKEY (11, 12) has shown that this characteristic appears also in early-ripening varieties of the sweet cherry (*Pr. avium* L.) and the sour cherry (*Pr. cerasus* L.). Furthermore, there is a relation between development of the pericarp and development of the embryo (12, 13, 14).

The pericarp of the peach develops in three stages (5, 6, 13). Stage I is characterized by rapid increase in size for a fairly definite period following bloom (49 to 53 days in 1934), and is of relatively uniform duration for all varieties, regardless of season of fruit ripening. In stage II development of the fruit is retarded for a variable period, depending on the variety. For a variety which ripens early (91 days from full bloom) this period is short, 5 days for Greensboro; while for a variety which ripens late (144 days from full bloom) it is long, 42 days for Chili. In stage III, known as the final swell, the fruit increases rapidly in size for a period which extends from the termination of the stage of retarded development until final ripening. The difference between an early-ripening and a late-ripening variety of peach, therefore, is largely a matter of duration of stage II.

Development of the embryo does not parallel that of the pericarp. During stage I the embryo develops slowly, so that 49 to 50 days after full bloom it is still microscopic. At the beginning of stage II, however, the embryo enters a period of rapid development which continues until it reaches maximum size for the variety. In the case of a very early-ripening variety, stage III is initiated while the

<sup>1</sup> Journal Paper no. 174 of the New York State Agricultural Experiment Station.

embryo is in this period of rapid development. The embryo fails to reach maximum size, the nucellus and integuments collapse, and a shriveled or abortive seed results. In the case of a late-ripening variety, the beginning of stage III does not occur until the embryo is nearly full size. Such seeds are viable, and will germinate following a period of after-ripening.

Embryos of early-ripening varieties often seem flaccid even though appearing to fill the integuments, and they may shrivel when excised and exposed. In contrast, embryos from most late-ripening varieties are firm and do not shrivel.

Such differences in texture at once suggest differences in composition. LOTT (8) has analyzed peach seed at different stages in development within the fruit and has shown it to undergo marked chemical changes. His analyses, however, did not include separation of the seed into its constituent parts, as embryo, endosperm, nucellus, and integuments.

The work reported in this paper was undertaken to determine the changes which occur in embryos during development within the seed, to compare the development and chemical composition of embryos of varieties ripening at different dates, and to throw light upon related problems of fruit, seed, and embryo development.

#### Material and methods

Ten varieties of peaches of different seasons of fruit ripening, extending from the early Greensboro which ripens August 3 to the late Salwey which ripens October 16, were selected for study. Fruits of these varieties were collected at or near the time of ripening, and the embryos examined and analyzed. Fruits of Greensboro, Governor Hogg, Early Rose, Triumph, and Rosebud were collected August 23, several days after the fruit was ripe. Fruits of Mamie Ross, Rochester, Lola, Elberta, and Salwey were collected when ripe. In addition, fruits of Elberta were collected during the growing season, and embryos analyzed at five periods, namely, July 10, July 21, July 31, August 25, and September 12, thus affording a comparison between the development and composition of embryos of early-ripening varieties and that of embryos of a late-ripening variety on the same date.



In preparing the material, seeds were removed from the fruits, and the integuments, remaining nucellus, and endosperm dissected away, leaving only the embryos. These were immediately weighed and divided into three samples for determination of fat, nitrogen, and sugars. The samples for fat (ether extract) were dried at 95° C. for two hours and then placed in a vacuum oven at 70° C. until they reached constant weight. The percentage of moisture was determined from these samples. The samples for sugar were placed directly in 80 per cent alcohol.

Fat was determined by the common ether extraction method; sugars by the method involving extraction by 80 per cent alcohol (1); and sucrose after inversion with invertase. The Munson and Walker method by direct weighing was used for all sugars. The determinations for reducing sugars were calculated as glucose. For nitrogen the Gunning method was used (1), excepting in some cases where the amount of sample available was limited, when the semi-micro-Kjeldahl method of UMBREIT and BOND (15) was employed.

Calculations were made on both the fresh weight and the unit basis, but since there was no appreciable difference in results, the fresh weight results alone are given.

### Results with Elberta

The condition and chemical analyses of embryos of Elberta taken at different stages in development are given in table 1. Seeds examined in the first sampling, July 10, contained embryos which were approximately one-third the length of embryos in mature seeds and were increasing rapidly in size. The integuments had already reached maximum size. Such seed, when removed from the fruit and exposed in the laboratory, promptly lost moisture. Subsequently the nucellus and integuments collapsed upon the embryo, giving the appearance of abortive seed. Successively later samplings showed increasingly larger embryos, a nearer approach to complete filling of the integuments, and less flaccidity. Mature seeds of this variety contained firm embryos which completely filled the integuments.

The moisture content decreased steadily from earliest to latest samplings. Fat increased during the same period. Moisture, however, was lost more rapidly than fat was accumulated, so that the

change was not merely replacement of water by fat. Other materials were also accumulating. Nitrogen increased slowly but steadily. Reducing sugars were found only as traces in the earliest sampling and remained at a relatively low level thereafter, reaching a maximum during the period of greatest accumulation of storage materials, and then declining as the time of fruit ripening approached. Sucrose appeared in small quantities in the earliest sampling and then increased with successively later samplings. No starch was found.

TABLE 1

CHEMICAL ANALYSES AND STAGES OF DEVELOPMENT OF EMBRYOS OF ELBERTA PEACH AT DIFFERENT STAGES DURING SEASON.  
FRESH WEIGHT BASIS

DATE OF SAMPLING	LENGTH (MM.)	DEGREE OF FILLING	CONDITION	PERCENTAGE					
				MOIS- TURE	ETHER EX- TRACT (FAT)	NITRO- GEN	CALCU- LATED PRO- TEIN (N X 6.25)	SU- CROSE	REDUC- ING SUG- ARS AS GLU- COSE
July 10..	6.5	One-third	Flaccid	93.10	0.37	0.50	3.13	0.14	Trace
July 21..	17.5	Nearly full	Flaccid	91.98	0.40	0.68	4.25	1.06	0.25
July 31..	19.0	Full	Flaccid	86.24	2.93	1.10	6.88	0.99	0.14
Aug. 25..	19.0	Full	Slightly flaccid	58.72	18.88	2.64	16.50	1.26	0.96
Sept. 12..	19.0	Full	Firm	45.63	30.67	2.60	16.25	2.32	0.23

#### Results with varieties ripening at different dates

The composition and characteristics of the embryos of nine varieties at the time of fruit ripening of each variety are given in table 2. In the case of the earliest-ripening variety (Greensboro) the embryos had aborted at such an early stage, were so small, and had disintegrated so badly, that sufficient material could not be secured for chemical analysis.

The moisture content declined steadily from the earliest ripening to each successively later-ripening variety. At the same time the fat increased steadily, following a linear curve, up to about 30 per cent, remaining fairly constant thereafter. Nitrogen likewise in-

creased in varieties ripening successively later, but more slowly. Sucrose appeared in the earlier varieties and remained at a nearly constant level in later-ripening varieties. Reducing sugars were not present in the embryos of the earliest-ripening varieties, were found only in traces in later varieties, reached a constant although fairly

TABLE 2

CHEMICAL ANALYSES AND STAGES OF DEVELOPMENT OF EMBRYOS OF PEACH VARIETIES RIPENING AT DIFFERENT DATES. FRESH WEIGHT BASIS

VARIETY	DATE OF FRUIT RIPENING (1936)	ABORTION (%)	DEGREE OF FILLING	CONDITION	PERCENTAGE				
					MOISTURE	ETHER EXTRACT (FAT)	NITROGEN	CALCULATED PROTEIN (N X 6.25)	SUCROSE
Greensboro....	Aug. 3	100	Half	Disintegrated	.....	.....	.....	.....	.....
Governor Hogg	Aug. 4	38	Two-thirds	Flaccid	85.25	1.14	.....	.....	1.70 0.0
Early Rose....	Aug. 5	40	Two-thirds	Flaccid	81.24	2.40	1.34	8.38	..... 0.0
Triumph.....	Aug. 7	15	Five-sixths	Flaccid	76.98	4.83	.....	.....	3.93 Trace
Rosebud.....	Aug. 16	0	Nearly full	Flaccid	69.89	7.97	1.66	10.38	4.32 0.40
Mamie Ross...	Aug. 23	4	Full	Flaccid	64.72	13.42	1.63	10.19	3.47 0.21
Rochester....	Aug. 25	0	Full	Slightly flaccid	63.19	14.97	1.77	11.06	3.31 1.18
Lola.....	Aug. 26	2	Full	Firm	60.14	15.86	1.73	10.81	3.60 0.25
Elberta.....	Sept. 12	0	Full	Firm	45.63	30.67	2.60	11.25	2.32 0.23
Salwey.....	Oct. 16	0	Full	Firm	38.95	29.82	3.13	19.56	3.70 0.19

low level in later-ripening varieties, and then declined slightly in the latest-ripening variety. No starch was detected in any of the samples, either by microscopic examination or by treatment with diastase.

A relation appeared between decreasing amounts of moisture and increasing amounts of fat in successively later-ripening varieties. In the earliest-ripening varieties the comparative decrease of moisture was greater than the increase of fat. In varieties ripening a little later the decrease in moisture was accompanied by an equivalent

increase in fat, and in the latest-ripening varieties the still smaller amount of moisture was not accompanied by a greater amount of fat. No doubt the embryos of earlier-ripening varieties would in time have lost moisture, likewise. Such a loss was not detected because analyses were made promptly after fruit ripening.

The fact that the smaller amount of moisture in the latest-ripening variety was not accompanied by a greater amount of fat may be due to the embryos having reached maximum development considerably before the fruit was ripe. Since the sampling was done at fruit maturity, the embryo may have lost moisture during this interval.

### Discussion

Growth of the embryo of the Elberta variety is plotted in figure 1, together with the moisture, fat, and nitrogen content. The graph shows clearly that the embryo reached nearly maximum size, as measured by length, before any appreciable accumulation of storage materials began. The loss of moisture and the accumulation of fat and nitrogen follow a linear curve for the most part.

Accordingly, when the embryo was making greatest increase in size the moisture content was high and the concentration of storage materials was low. As the embryo approached maximum size, accumulation of sucrose began, followed in turn by loss of moisture and accumulation of fat. Inasmuch as the nitrogen occurs largely as glucosides and proteins, the calculated protein values more nearly approximate the total quantity of nitrogenous substances actually present in the embryo than do the figures for nitrogen alone. The fat and protein together comprise 41 to 49 per cent of the fresh weight at maturity. The results are similar to those reported by FINCH (4) and by THOR and SMITH (10) for the development of the pecan kernel, and by REEVES and BEASLEY (9) for the development of the cotton embryo.

The data dealing with the other nine varieties show a definite trend and relationship between varieties. Thus in figure 2, where moisture, fat, and nitrogen content of each variety are plotted against time of fruit ripening, the resulting graph appears similar to that for the development of a single variety (Elberta) throughout the season (fig. 1).

The entire situation suggests a steady increase in growth, loss of moisture, and accumulation of storage materials in all varieties, and

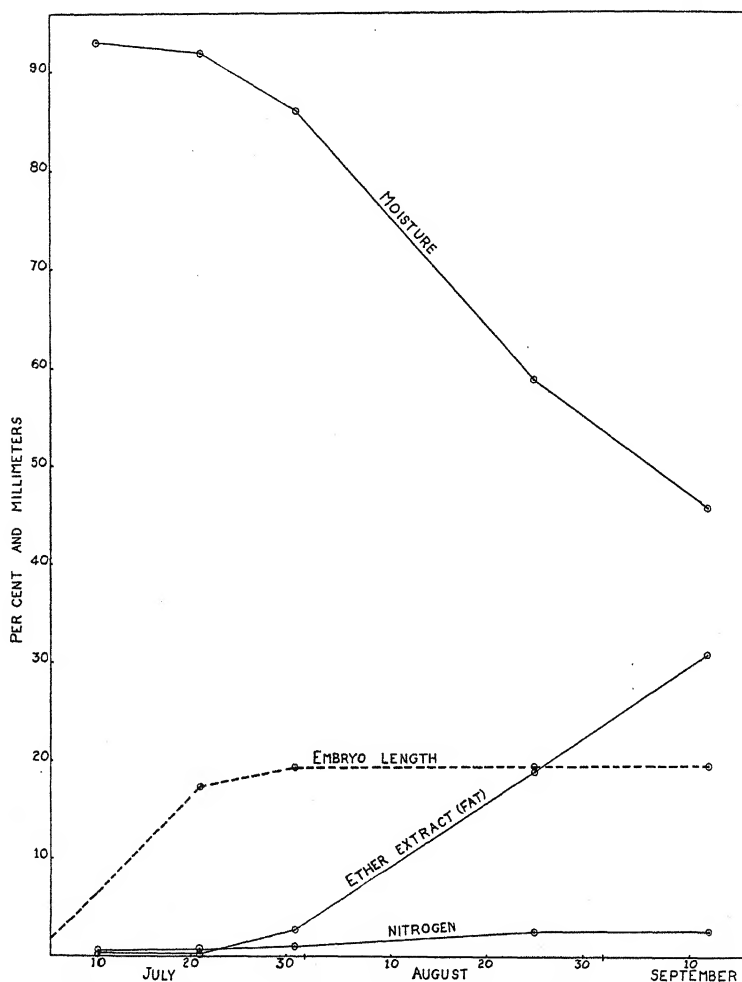


FIG. 1.—Growth of embryo of Elberta peach in relation to loss of moisture and accumulation of fat and nitrogen during development.

an abrupt check in these processes as the successive ripening dates for the different varieties are reached.

It has already been observed (13, 14) that the rate and duration of

the growth periods of peach embryos are strikingly similar regardless of wide differences in season of fruit ripening. That is, during the

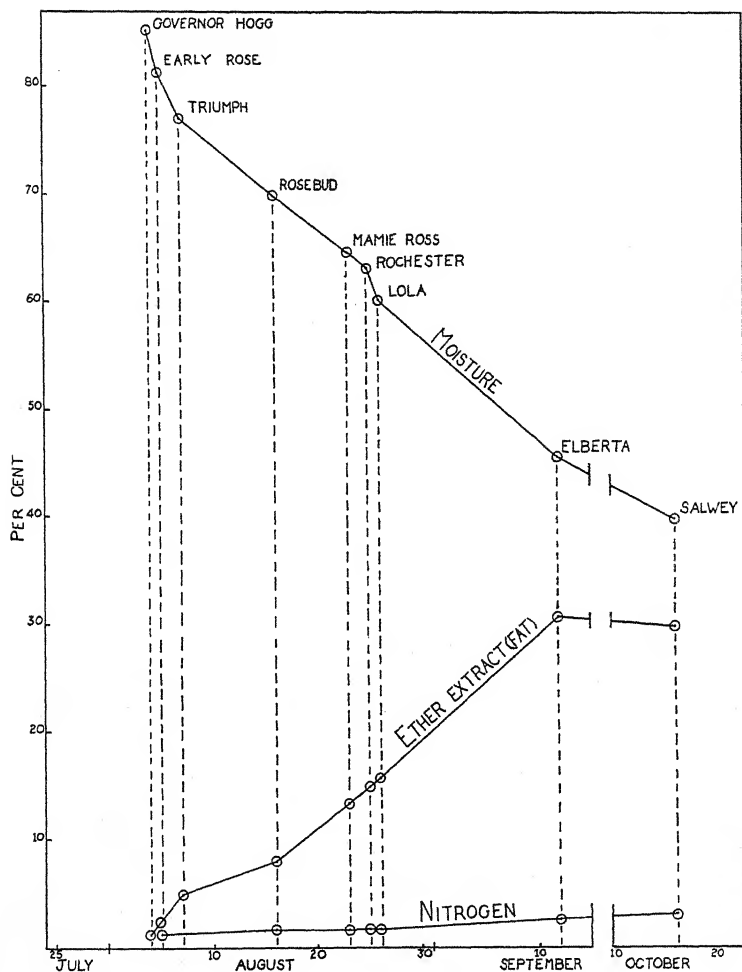


FIG. 2.—Comparison of moisture, fat, and nitrogen content of nine varieties of peaches at time of fruit ripening (*cf.* fig. 1).

season of 1934 with varieties ripening as widely apart as 91 and 144 days after full bloom, embryos of all varieties developed slowly for a period of 50 days following full bloom and then abruptly entered a

period of rapid development. A similarly striking relation is seen to exist in the chemical changes within the embryo.

The abruptness of the check in rapid accumulation of storage materials near the time of fruit ripening indicates a sharp interference with the supply of materials. The data suggest either an interruption in the vascular structure supplying the embryo or a shifting of the supply of nutrients from the embryo to some other portion of the plant. Such a shift might be thought of as being brought about by an accelerated metabolic activity in some other part of the plant, by a change in nutritional balance within the embryo, by altered enzyme activity, or by the appearance or disappearance of some growth substance. The controlling mechanism is of vital interest in connection with embryo abortion and fruit ripening in early-ripening varieties of stone fruits, and merits further study.

The relatively high moisture content of embryos of early-ripening varieties helps to explain the manner in which seeds of these varieties become shriveled, for not only may a seed shrivel because it contains an abortive embryo which never completely fills the integuments, but also because an embryo which does completely fill the integuments may later lose moisture and shrink. The relatively lower fat, nitrogen, and sucrose content of embryos of early-ripening varieties is also of interest. It will be recalled that the gross structure of the embryo is completed at a relatively early date, when accumulation of these materials and particularly of fat is low. For example, in the development of Elberta, the embryo had reached maximum size for the variety and completely filled the integuments 43 days before the fruit was ripe, yet the fat content was only 2.93 per cent as compared with 30.70 per cent at fruit ripening. In contrast, embryos of an early-ripening variety (Rosebud) which nearly filled the integuments at fruit ripening contained only 7.97 per cent fat.

LILLELAND and NEWSOME (7) describe similar results for the Elberta in California. They report that when the developing embryo is 90.6 per cent of maximum length for the variety, the fat content of the seed is only 8 per cent of what it is at maturity. In discussing the development of the cherry they say further (7) that tentative data on fat analyses of seeds of the Early Purple Guigne, Chapman,

and Mazzard indicate that the three early-ripening kinds are considerably lower in fat than the later-ripening Mazzard. They suggest that such data show that maturity of the seed of the cherry as measured by increase in fats and total solids does not take place until after the embryo has made its major length growth, and that in the early varieties this may lead to a seed low in fat and lacking viability. Yet they do not consider such seed to be abortive, and accept an embryo as abortive only "when examination reveals premature cessation of growth or evidence of disintegration *while the fruit is on the tree.*"

Using this concept, they have examined seed of two early-ripening and four later-ripening varieties of sweet cherry. Although they interpret the data to show that early-ripening cherries, as grown at Davis, California, do not have abortive embryos, yet they show that Early Purple Guigne and Chapman, the two earliest-ripening varieties of the six which they studied, produce 5.7 and 9.3 per cent abortive seed, respectively, while for three of the four later-ripening sorts (Lambert, Downer, and Lewellyn) they report 0.0 per cent abortive seed.

From the data presented by them there can be no doubt that the embryos of their Early Purple Guigne developed to larger size in California than do embryos of that variety in New York (12). Yet the apparent disagreement between the data in California (7) and those in New York would seem to be mostly one of degree and of definition. In the essentials they appear to be in entire agreement.

Using the word abortive in its meaning of checked or imperfectly developed, an embryo which fills the integuments while on the tree, but which fails to develop storage materials, is just as truly abortive as one which has failed to fill the integuments. The fact that accumulation of storage materials lags behind increase in size does not eliminate the accumulation of stored products from being a part of full functional development.

### Summary

1. Embryos of ten varieties of the peach, whose several seasons of fruit ripening extend from early to late, were examined and chemically analyzed at or near the time the fruit was ripe. For compari-



son, embryos of a single late-ripening variety (Elberta) were analyzed periodically throughout the season.

2. Embryos of Elberta were flaccid and only partially filled the integuments during stage II of fruit development, so that seed became shriveled when removed from the fruit. At the time of fruit ripening, embryos were firm and completely filled the integuments.

3. Embryos of the earliest-ripening variety were abortive and disintegrated at fruit ripening. Embryos of each successively later-ripening variety were larger, firmer, and more nearly filled the integuments.

4. At fruit ripening, embryos of a late-ripening variety contained 45.03 per cent moisture, 30.67 per cent fat, 2.60 per cent nitrogen, 2.32 per cent sucrose, and 0.23 per cent reducing sugars. Embryos of early-ripening varieties showed a higher moisture content and lower content of fat, nitrogen, reducing sugars, and sucrose.

5. There is a steady increase in growth, loss of moisture, and accumulation of storage materials in embryos of all varieties, and an abrupt check in these processes as the ripening date for each successive variety is reached.

6. If the moisture, fat, and nitrogen content of the embryos of the nine varieties are plotted with reference to the time of fruit ripening, the composite graph so formed is similar to that for the development of a single variety.

7. The close similarity already observed in growth of embryos in different varieties, regardless of season of fruit ripening, extends to chemical changes within the developing embryos as well.

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## HYDATHODES IN THE GENUS EQUISETUM

MARION A. JOHNSON

(WITH EIGHT FIGURES)

### Introduction

BURGERSTEIN (1) has reported the occurrence of guttation in 333 genera from 115 widely distributed families. It is not so well known, however, that *Equisetum* is a convenient and suitable subject for demonstrating the secretion of water. *Equisetum scirpoides*, *E. hiemale*, *E. variegatum*, and *E. arvense*, all of which may be readily grown in the greenhouse, are satisfactory for this purpose. When well watered plants are placed under a bell jar, conspicuous drops soon appear on the leaves and sheaths. This phenomenon is most pronounced in young shoots but it may also be seen in mature ones. *E. scirpoides* has not only proved to be suitable for illustrating guttation but is an easily available source from which samples of the liquid may be collected for chemical analysis.

ROBERT (4), apparently one of the earliest to record the occurrence of guttation in *Equisetum*, mentions that *E. arvense* and *E. fluviatile* secrete water which appears in prominent drops on the leaves. Later VOLKENS (9), in a paper devoted to the secretion of water in the higher plants, states that water drops are formed on the leaf and sheath of *E. arvense* and *E. limosum* and that they are secreted by stomata located on the inner face of the leaf sheath. Since then SCHAFFNER (6) has reported guttation for *E. praealtum*, *E. hiemale*, and *E. variegatum*. So far as the writer is aware, no adequate descriptions or figures of the hydathodes in *Equisetum* have appeared in the literature, except for the brief reference made to them in an earlier paper by JOHNSON (3) on *E. scripoides*. The species shown on the following page, including practically all of the living species of the genus, and arranged according to SCHAFFNER'S (5) classification, have been studied.

The material examined consisted of leaves and leaf sheaths from both herbarium and living specimens. These were cleared for sev-

eral days in a saturated solution of chloral hydrate and examined *in toto*; in addition, thin sections were made by the paraffin method and stained in safranin and fast green.

## EQUISETA PRIMITIVA

*E. xylochaetum* Mett.<sup>1</sup>

*E. giganteum* L.<sup>1,4</sup>

## EQUISETUM HIBERNA

*E. ramosissimum* Desf.<sup>1</sup>

*E. debile* Roxb.<sup>1</sup>

*E. laevigatum* A.Br.

*E. praealtum* Raf.

*E. moorei* Newm.<sup>1</sup>

*E. hiemale* L.

var. *californicum* Milde.<sup>2</sup>

## EQUISETA PUSILLA

*E. nelsoni* (A.A.Eat.)

Schaffn.<sup>1</sup>

*E. trachyodon* A.Br.<sup>1</sup>

*E. variegatum* Schleich<sup>1</sup>

*E. scirpoides* Michx.

## EQUISETA AESTIVALIA

*E. fluviatile* L.

*E. palustre* L.

*E. bogotense* H.B.K.<sup>1</sup>

*E. diffusum* D.Don.<sup>1</sup>

## EQUISETA AMBIGUA

*E. kansanum* Schaffn.<sup>3</sup>

*E. funstoni* A.A.Eat.<sup>2</sup>

## EQUISETUM HETEROPHYADICA

*E. silvaticum* L.

*E. pratense* Ehrh.

*E. telmateia* Ehrh.

*E. arvense* L.

## Observations

Hydathodes were found in each of the twenty-two species examined and probably occur in every species in the genus. They are confined to a restricted area on the adaxial surface of the leaves or sheath, or on both, depending upon the species. This region (figs. 1-3), which is considerably lighter in color than the adjacent chlorophyllose tissue and which is always intimately associated with the adaxial face of the foliar vein, constitutes a single hydathode. In *E. variegatum*, *E. palustre*, *E. ramosissimum*, and *E. giganteum*, where the leaves are discolored, it can readily be recognized by the absence

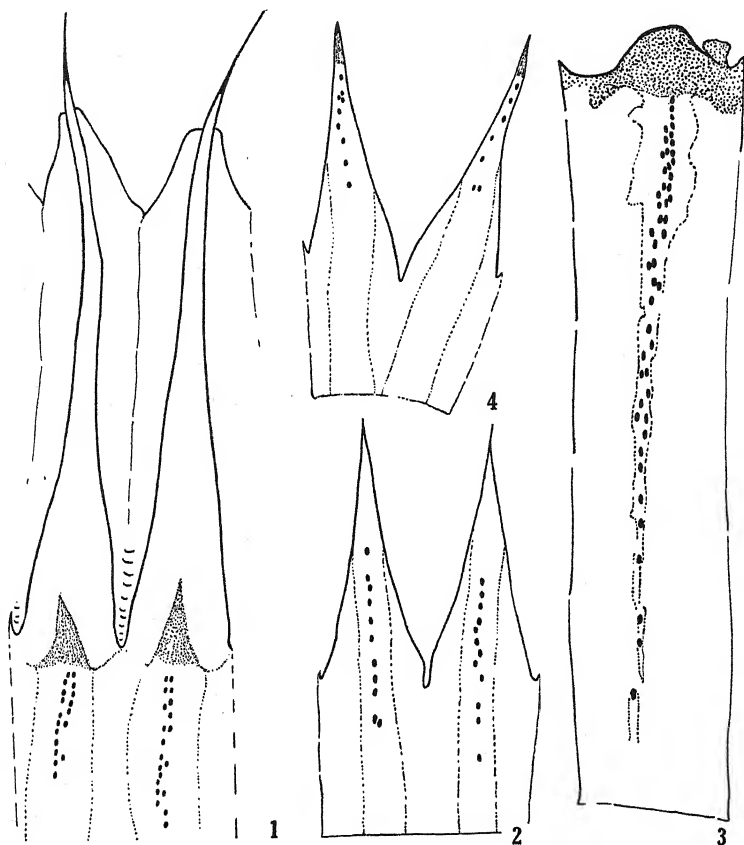
<sup>1</sup> Obtained from the New York Botanical Garden through the courtesy of the Director.

<sup>2</sup> Supplied by Dr. A. W. HAUPT of the University of California at Los Angeles.

<sup>3</sup> Furnished by Dr. ELDA R. WALKER of the University of Nebraska.

<sup>4</sup> Collected in Mexico by Dr. C. J. CHAMBERLAIN of the University of Chicago and in Jamaica by Dr. M. A. CHRYSLER of Rutgers University.

of coloring matter in its epidermal cells. The hydathodes are much longer than broad and are studded with pores resembling stomata in general appearance but differing from them in their position above a



FIGS. 1-4.—Fig. 1, *E. hiemale* var. *californicum*: leaves and sheath showing position of water stomata in hydathode. Fig. 2, *E. arvense*: same from main axis of aerial shoot showing position of water stomata in hydathode. Fig. 3, *E. giganteum*: sheath from main axis showing position of water pores in hydathode. Fig. 4, *E. arvense*: leaves and shoot from ultimate lateral branch of aerial shoot showing position of water pores in hydathode.  $\times 16$ .

vein. The average length for the hydathodes throughout the genus is about 1 mm. They are, however, longer in the large leaves; for instance in *E. giganteum* they are often 5 mm. long (fig. 3), and may

extend for a distance of 15–20 mm. on the leaves of the fertile shoot of *E. telmateia*.

Studying the distribution of the hydathodes throughout the genus, it was found that they either extend well up into the leaves (figs. 2, 4) or are confined to the sheath and leaf bases (figs. 1, 3). The former condition is seen in the *Equiseta Aestivalia* and *Equiseta Heterophyadica*, where the leaves are persistent, contain considerable photosynthetic tissue, and do not dry out at maturity; while the latter occurs in the *Equiseta Primitiva*, *Equiseta Hiberna*, and *Equiseta Ambigua*. The species in the last mentioned three sections have deciduous leaves and thus the basal position of the hydathodes permits their retention when the leaves are shed. This arrangement may be of value to the species in question, yet the position of the hydathodes appears to be correlated with the thickness of the leaves rather than with their deciduous nature. The *Equiseta Pusilla* bear out this hypothesis, for here the leaves are not only characterized by their extreme thinness and early desiccation, as in the *Equiseta Primitiva*, *Hiberna*, and *Ambigua*, but also by being persistent; and it is significant that the hydathodes are confined to the sheath and leaf bases just as they are in the species with deciduous leaves.

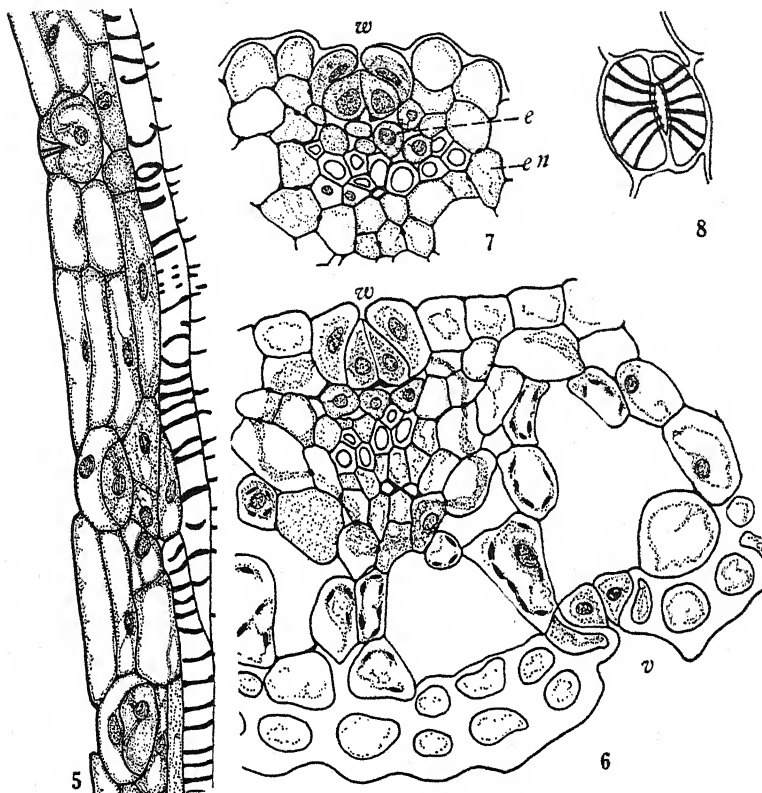
A detailed study of the distribution of the hydathodes in *E. arvense* shows them to be present on the leaves of both the sterile and fertile shoots, and of the rhizome as well. One notable exception is the case of the ocreole or first sheath of the branches, where they are absent. This is not surprising in view of the reduced condition of the ocreole, for it is only a few cells in thickness and may be devoid of vascular tissue. Sometimes veins are differentiated, however, but even so hydathodes are not developed. The hydathodes on the rhizomes are definitely restricted to the sheath and base of the leaves and measure from 1 to 1.5 mm. in length, while those from near the apex of the sterile shoots are confined to the leaf tips and are reduced in length to about 0.5 mm. The gradual decrease in length and the restriction of the hydathodes to the distal end of the leaves parallel the reduction in leaf size seen in passing from the rhizome to the ultimate nodes of the sterile branches. This relationship between leaf size and length of hydathodes is clearly shown in the large conspicuous leaves of the fertile shoot of *E. telmateia*, where they may be 15 to 20 mm. in length.

Evidence is not at hand to show whether the hydathodes from the rhizomes are functional. They are a constant feature in *E. arvense* and *E. silvaticum*, however, but even in these species those more than three nodes below the soil level have imperfectly developed water pores, so that there is some doubt as to their ability to secrete water. In *E. scirpoides* and *E. praealtum*, hydathodes were not observed from the rhizomes, and unfortunately suitable material was not available for further study concerning this point in the remaining species.

Longitudinal and transverse sections through the leaves (figs. 5-7) show that in *Equisetum* the hydathodes are simple in structure. At most they consist of a few layers of epithem tissue derived from the pericycle on the adaxial face of the xylem, an endodermis which is extended until it joins the epidermis, and a varying number of water pores opening upon the adaxial surface of the leaf or sheath throughout the length of the hydathode. Occasionally the epithem tissue is so poorly developed that a lignified cell of the xylem lies exposed beneath a water pore. Dense cytoplasm, large nuclei, few if any chloroplasts, and thin cellulose walls characterize the epithem cells. They are also considerably shorter than the cells which lie in a corresponding position below the hydathode and their arrangement allows for small intercellular spaces in the vicinity of the water pores (figs. 5-7).

It has been assumed thus far that the areas described as hydathodes really do function in the secretion of water, and there is evidence supporting this assumption. When the hydathodes are conspicuously located at the tip or edge of the leaves, as is the case in the Crassulaceae, it is relatively easy to trace the drops of water from guttation to their source. In *Equisetum*, however, where the leaves are small and closely appressed to the stem this is not so readily accomplished. Here the water collects above the node between the leaf and stem. Thus there are two natural openings from which it might have escaped, the stomata on the stem immediately above the node and the pores on the adaxial face of the leaf and sheath. Considering the first possibility, it can be stated with certainty that the stomata are not underlaid with secretory tissue, that they are not in close proximity to the vascular bundles, and that they are

separated from the vascular tissue by a well developed endodermis. These stomata are not adapted for guttation and it is extremely doubtful whether they function in this capacity. On the other hand, the pores in the hydathodes are located above the veins, are in direct



FIGS. 5-8.—Fig. 5, *E. arvense*: structure of hydathode in longitudinal section. Fig. 6, *E. kansanum*: hydathode and ventilating stoma in transverse section; note that endodermis joins epidermis (*w*, water pore; *v*, ventilating stoma). Fig. 7, *E. arvense*: hydathode in transverse section (*w*, water stoma; *e*, epithem tissue; *en*, endodermis). Fig. 8, *E. palustre*: surface view of guard cells of a water stoma.  $\times 375$ .

communication with a distinct epithem tissue which in turn is in contact with the xylem, and the whole unit is surrounded by an endodermis. The entire arrangement is not only well suited for the



secretion of water but would prevent the adjoining mesophyll tissue from becoming flooded. These facts are considered as sufficient reason for regarding the pores associated with the veins, and not the ventilating stomata, as the avenues through which the water is secreted.

The number of water stomata in a hydathode varies from three to four in the small branches of *E. silvaticum*, where the leaves are much reduced, to as many as fifty in *E. giganteum*, and the number may be even higher in the large leaves on the fertile shoot of *E. telmateia*. A comparison of the water stomata with the ventilating stomata shows them to be similar in general plan but with the former generally somewhat smaller in size, as seen in surface view. Both are derived from epidermal initials cut by two slightly oblique radial walls into three cells whose long axes parallel that of the leaf. The angle at which the walls are formed makes the central cell broadest on its inner tangential face while the flanking cells are broadest on the outer tangential faces. The central cell, upon division, gives rise to the guard cells bearing radial markings; these at maturity are overgrown by the flanking or accessory cells. Neither the accessory nor the guard cells are displaced as much as are those of the ventilating stomata, and in cross section they appear more nearly at right angles to the epidermis. There is, however, considerable variation in respect to this point (figs. 6, 7). The chief differences between the two types of stomata are to be seen in their contents and in the thickness of their cell walls. In no instance could chloroplasts be found in the guard cells of the water stomata; this point was verified from both living and stained preparations. The walls of the guard cells of the water stomata are much thinner than those of the ventilating stomata and are more nearly uniform in thickness, except for a slight thickening on the inner tangential face (figs. 6, 7).

Continuing a comparison of the water pores and ventilating stomata, it will be recalled that in the *Equiseta Aestivalia* and *Heterophyadica* the stomata are not sunken below the surface of the epidermis as they are in the remaining four sections of the genus. The water pores, however, are flush with the surface or are very slightly sunken throughout the genus (figs. 5-7). This feature is remarkably constant, regardless of whether the species are mesophytes or xerophytes, and is to be added to the list of characteristics showing little

variation in this old and stable genus. The position of the water pores on the upper surface of the appressed leaves offers considerable protection against desiccation, and may in part be responsible for the failure to develop the sunken habit in the xerophytic species.

The absence of chloroplasts and the uniform thickness of the radial walls of the guard cells raise the question as to whether it is possible for the water stomata to be opened or closed to any great extent. Examination of material preserved in formalin-acetic-alcohol generally shows the guard cells to be open (fig. 8), but in living material they appear to be closed. This point was investigated with the aid of a Leitz Ultrapak. Leaves from young stem tips of *E. praealtum* were removed while actively secreting water and mounted in water at once; some of the guard cells were slightly open but for the most part they were entirely closed.

#### Discussion

So far as the writer is aware, the epithem type of hydathode has always been described as being intimately associated with the free tip of tracheids, representing either the distal end of a foliar veinlet or modifications from the nodal points of the vascular network. The former condition may readily be seen in *Primula sinensis* Lindl., where the veins of the leaf teeth abut directly upon the epithem tissue, and as HABERLANDT (2) has clearly shown, some of the tracheids may even project into the intercellular spaces. The second type is illustrated by the prominent hydathodes in *Crassula quadricifida* Baker, which are supplied by tracheids arising from the tip of the veins and spreading out in the form of a fan before entering the epithem. In *Equisetum* the hydathode differs from either of these types; here the epithem is confined to the face of the vein and does not extend to its tip. The tracheids are not displaced from their course but continue undisturbed throughout and even beyond the length of the hydathode. This places the epithem cells and their intercellular spaces in direct contact with the long axis of the tracheids, and judging from the amount of water secreted, is an efficient arrangement. This type of epithem hydathode is apparently unique.

Phylogenetically water stomata are regarded as having originated from ventilating stomata. Whether this could apply in *Equisetum* was a question until stomata were found on the adaxial face of the

leaves in *E. telmateia* and *E. diffusum*. Those of the former are confined strictly to the fertile shoots, where they extend from the base of the sheath to within 15-20 mm. of the leaf tips. The aerial shoots in *E. diffusum* are not separated into sterile and fertile shoots, but instead the main axis is terminated by a cone, with the lower nodes producing sterile branches while the upper ones have none. Only the leaves from the upper nodes, and especially from the final node nearest the cone, have stomata on the adaxial face. It is significant that in each of these species adaxial stomata are associated with reproductive structures which in general are noted for their conservative nature. The discovery of these stomata in *E. telmateia* and *E. diffusum* is of considerable importance, in that it may be taken to indicate that *Equisetum* or its ancestors once had stomata on the upper surface of their leaves and that from them the water stomata of the hydathodes have been derived.

The wisdom of searching the fossil record of *Equisetum* for evidence in support of this hypothesis is obvious, for as SCOTT (7) has remarked, "In fact, we may safely say that any adequate knowledge of the Equisetales must be derived to a much greater extent from the study of the extinct forms than from that of the few surviving representatives." An examination of the Paleozoic Calamites whose equisetaceous affinities are undoubted shows stomata on the adaxial face of the leaves. As in *Equisetum*, the foliage was borne in verticils but the free tips were often more extensively developed, as is to be seen to good advantage in the linear, lanceolate, or spatulate leaves of *Annularia*. Unfortunately the internal structure of the foliage of *Annularia* is unknown. In *Asterophyllites* the leaves were linear or lanceolate and resembled those of *Equisetum*, except that they were not united into a sheath at the base. The anatomy of the *Asterophyllites* type of leaf has been investigated by THOMAS (8), who describes each leaf as being supplied with a vascular bundle which was surrounded on all sides by photosynthetic tissue. In the tip of the leaves fibers instead of parenchyma occurred above the veins. Unlike *Equisetum*, the stomata were restricted to the upper surface of the leaves, where they occurred in rows but with a tendency to be absent above the veins. No mention is made of structures which might be interpreted as hydathodes. Negative evidence, however, does not preclude the possibility of their occurrence among the Cala-

mites, especially when it is realized that the leaf structure is known for relatively few species of this great group of plants.

Taking the Calamite leaf as the general ancestral type from which the *Equisetum* leaf was derived, the origin of the hydathodes may be visualized as having occurred in the following manner. The leaves were reduced in size and the photosynthetic tissue was limited to the abaxial face of the leaf, while the vein approached the adaxial face. These changes were accompanied by a restriction of the stomata to the thicker parts of the leaf. Thus those on the adaxial face were confined to the region above the foliar vein and new ones were initiated on the abaxial face in connection with the photosynthetic tissue, as is seen in *Equisetum* today. Eventually in the leaf tips the vein touched the epidermis and the endodermis became joined to the epidermis by a Casparian strip, thereby isolating the adaxial stomata from the remainder of the leaf. A slight proliferation of the pericycle, together with the loss of the chloroplasts in the guard cells, would produce the epithem hydathode of *Equisetum*.

These stages are practically duplicated in the leaves of the fertile shoot of *Equisetum telmateia*. The adaxial face is supplied with several rows of stomata at the base, while at the tip where the leaf is much thinner they are generally reduced to a single row lateral to each side of the vein. The endodermis of the vein is continuous with the epidermis throughout the length of the hydathode, but below this point there are stomata which are not connected with the vein. In all probability these represent ventilating stomata that have been isolated above the vein during the evolutionary process.

The characteristics exhibited by the hydathode are apparently of no value in determining the degree of relationship between the species of *Equisetum*. Their structure is extremely simple and remarkably uniform throughout the genus. The most noticeable variations are in their position and in the number of water stomata. The former, however, depends upon the thickness of the leaves while the latter is correlated with leaf size regardless of the species.

### Summary

1. Hydathodes of the epithem type are reported for twenty-two species of *Equisetum*.
2. The hydathodes are confined to the adaxial face of the leaf and

sheath. They extend along the foliar vein but are not associated with the free tips of the vascular elements.

3. The epithem is derived from the pericycle of the vein and is incompletely developed.

4. Pores of the water stomata type connect the hydathodes with the surface of the leaf. Their number varies with the size of the hydathodes, which in turn is related to leaf size.

5. In species with very thin or deciduous leaves the hydathodes are borne on the sheath or leaf base, while in those with persistent and relatively thick leaves they are borne on the leaf and often near the tip.

6. Throughout the genus the water stomata are on a level with the surface of the epidermis.

7. Phylogenetically the water stomata appear to be derived from ventilating stomata which have been isolated above the veins on the adaxial surface of the leaf and sheath. The presence of ventilating stomata on the adaxial face of the foliar members in *E. telmateia*, *E. diffusum*, and in certain of the Calamariaeae supports this hypothesis.

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## FORMS OF CRATAEGUS CRUS-GALLI

H. W. RICKETT

The Crus-Galli are the largest group of *Crataegi* in Missouri, more than forty species having been described. As usual in this genus, these numerous species are distinguished by only small differences, often in such variable characters as shape and size of leaves. Most of them were described by SARGENT,<sup>1</sup> who later<sup>2</sup> was led by observations of PALMER to merge several of the supposed species. The question arises whether such an effort to reduce the multitude of named species to a more usable number may not conceal real racial differences and so obscure rather than help to elucidate the *Crataegus* problem. In a previous paper (*l.c.*) I have shown that proper methods of study reveal constant differences, not apparent to ordinary observation, between local races of *C. pruinosa* (Wendl.) Koch. A study of local forms of the Crus-Galli has yielded similar results.

In this complex group, which intergrades with the Punctatae and the Virides, can be distinguished a plexus of forms which differ only slightly among themselves and all of which are evidently closely related to *C. crus-galli* L. This species, interpreted as including a large number of such local races, may be briefly described as follows.

Shrubs, with long straight spines. Leaves coriaceous, lustrous, usually glabrous, from obtuse or rounded to acute, from obovate or spatulate to elliptic, 30-60 mm. long, about half as wide, on very short petioles (4-8 mm.). Leaves on non-flowering branches often larger and more acute, sometimes lobed, but in size and shape varying greatly from branch to branch and from year to year. Cymes of 10-20 flowers, usually glabrous; bracts deciduous before anthesis. Sepals linear, entire, or more or less gland-serrate. Stamens usually about 10 or (less often) 20. Anthers cream colored or pink. Styles 1-3. Fruit usually oblong, 8-14 mm. in diameter, more or less pruinose. Nutlets 1-3, 6-8 mm. long, usually strongly ridged dorsally; many lacking seeds.

<sup>1</sup> For references, see previous paper in BOT. GAZ. 97:780-793. 1936.

<sup>2</sup> Jour. Arn. Arb. 6:1. 1925.

Three forms of this species may be seen near Columbia, Missouri, differing only in number of stamens, color of anthers, and other less evident characters. Individual plants were marked and studied in successive years. Statistical studies of the numbers of stamens and styles have been attempted, but remain incomplete through a certain fatality which pursues marked trees; many of which have been felled or burned or otherwise killed, while others have failed to bloom. It seems probable, however, that the different numbers of stamens and of styles which characterize these forms are constantly associated with different colors of anthers. It is interesting that the few counts so far completed show a mean number of styles per flower in one form of 1.3; in another of 1.9; and these figures reappear in counts of the numbers of nutlets per fruit. Such analysis reveals a real difference between races which would otherwise be classed together as having "styles 1-2." Such characters are more reliable than many ordinarily used. Those of fruits, for instance, are in general so affected by the vagaries of climate and by the attacks of parasites as to be almost useless; in the forms under consideration the color of fruits varies from year to year on one plant from brick-red or bright cerise to dull crimson.

A series of measurements of spines yields a significant difference between plants classed in different groups according to their floral characters. This is shown in table 1. For purposes of comparison measurements are included also of spines of *C. danielsii* Palmer (see later) and of several of the *Punctatae* of this region. Measurements were made in the field on 25 spines per plant. Other measurements were made on herbarium specimens, the numbers varying from 20 to 50 per plant; these naturally are apt to represent flowering or fruiting branches more than the others, and it is therefore not surprising that the average of measurements made in the herbarium does not always coincide with that of measurements made in the field on the same plant. Such differences are certainly not significant; differences of similar magnitude between individual plants of the same group are likewise probably without meaning; but there is no question that one group is constantly characterized by shorter spines. As evidence of the reliability of the difference, it is worth recording that the classification by length of spines only of an un-

labeled plant was later found to be correct when floral characters were studied.

These data are interesting chiefly as they confirm those obtained from *C. pruinosa*; as in that species, the distinguishable characters

TABLE 1  
LENGTH OF SPINES (IN MM.) IN LOCAL RACES OF CRATAEGUS

CRUS-GALLI		
Anthers cream Stamens 7-10 Styles 1-2 (1.3)	Anthers pink Stamens 15-20 Styles 1-2	Anthers pink Stamens 7-10 Styles 1-2 (1.9)
Measured in the field		
(47)* $51.5 \pm 1.10$ (48) $51.3 \pm 1.34$ (37) $43.3 \pm 0.71$ (70) $43.9 \pm 0.90$	(65a) $48.1 \pm 0.10$ (69) $43.1 \pm 0.92$	(71) $30.0 \pm 0.97$ (60) $30.3 \pm 0.81$ (46) $27.1 \pm 0.99$
Measured in the herbarium		
(47) $42.5 \pm 0.77$ (48) $39.9 \pm 1.54$ (37) $43.2 \pm 0.91$ (70) $47.2 \pm 0.73$ (44) $53.1 \pm 1.70$ (41a) $51.9 \pm 1.37$	(65a) $40.1 \pm 1.05$ (69) $40.6 \pm 0.87$	(36) $30.7 \pm 1.30$ (60) $24.9 \pm 1.21$ (46) $28.9 \pm 0.52$
C. DANIELSII	PUNCTATAE	
(35) $33.5 \pm 0.76$ (8) $36.9 \pm 0.61$ (8)† $35.3 \pm 0.97$ (19a) $33.1 \pm 0.84$	(24) $49.3 \pm 1.20$ (25) $45.9 \pm 0.80$ (65) $43.1 \pm 1.41$	(16) $38.5 \pm 1.06$ (19) $37.9 \pm 1.09$ (20) $38.7 \pm 0.79$

\* Numbers in parentheses are those attached to individual plants for the purpose of recognition.

† Measurements made on this plant in two seasons.

are not combined at random in what CLAUSEN has called a "hybrid swarm," but are associated in a smaller number of constant combinations. The plants characterized by each of these combinations are supposedly related and capable of formal recognition. It is probable



that hybridization was involved in the origin of the numerous races of *C. crus-galli*, and probable also that their constancy and geographic restriction are results of apomictic descent from their hybrid ancestors.

It is unfortunately impossible to know whether or not these forms are identical with any of those previously described; such identification must await further studies of this kind. The form with cream colored anthers resembles *C. arduennae*, which has been identified (as already mentioned) with *C. albanthera*, *C. candens*, and *C. ferox*. The short-spined form with pink anthers and about 10 stamens resembles *C. hamata* and *C. infesta*. *C. strongylophylla* and *C. permera* also are similar except in having usually more styles. All of these forms seem to have more glandular sepals than the plants of this vicinity. The form with more numerous stamens seems to be nearly unique in this respect among pink-anthered forms of *C. crus-galli*.

Through the kind cooperation of J. M. GREENMAN, Curator of the Herbarium of the Missouri Botanical Garden, and of E. J. PALMER, of the Arnold Arboretum, I have been able to study the collections upon which SARGENT based his descriptions of the Crus-Galli of Missouri. It is difficult to interpret any particular specimens as types, but it is easy to find the specimens cited in the descriptions; and these are very uniform and closely resemble specimens from the same localities collected subsequently. Such characters as can be recognized (for instance, pubescence and margination of sepals) seem to provide constant means of differentiation between these "species." Even discounting conclusions based upon herbarium material and upon SARGENT's detailed descriptions, the results of the present study make it probable that at least most of these named races are really distinct.

It is with hesitation that I assign names to these forms. Their existence must be recognized and their characters catalogued, if the species which they compose is to be understood. But their number is so vast and their distribution so restricted that a less elaborate system is needed for their designation; letters or numbers, perhaps. Until definite action by botanists provides for such a situation, however, it is necessary to use the system now recognized. I have discussed elsewhere my reasons for regarding these races of *Crataegus* as

forms and the complex of forms as a species. I am here concerned only with naming the forms which I have carefully studied in the field; reduction of the multitude of already described "species" must await more exact characterization of them.

*Crataegus crus-galli* L. **vulgaris** f. nov. Folia cymique glabra. Spinae 40-50 mm. Stamina ca. 9, antheris eburnis. Styli 1-2 (1.3). Fructus lati 10-14 mm. Nuculae longae 6-7 mm. *Rickett* no. 46, May 5, 1931 (Herb. Univ. Mo. 8959).

*Crataegus crus-galli* L. **staminea** f. nov. Folia cymique glabra. Spinae 40-50 mm., saepe recurvatae. Stamina ca. 15-20, antheris roseis. Styli 1-2. Fructus lati 11-12 mm. Nuculae longae 6-7 mm. *Rickett* no. 750, May 21, 1935 (Herb. Univ. Mo. 17229).

*Crataegus crus-galli* L. **spinulosa** f. nov. Folia cymique glabra. Spinae 25-30 mm. Stamina ca. 10, antheris roseis. Styli 1-2 (1.9). Fructus lati 10-12 mm. Nuculae longae 6-8 mm. *Rickett* no. 47, May 20, 1931 (Herb. Univ. Mo. 8964).

*C. danielsii* Palmer also is evidently related to the preceding forms. It is locally very abundant, on rocky hills, in old pastures, and in vacant ground in Columbia. It is a handsome shrubby tree, usually loaded with blossom or fruit. The leaves are coriaceous and shining as in *C. crus-galli*, but more often acute and frequently lobed. Spines average about 35 mm. The cymes have a thin deciduous pubescence; each bears 15-20 flowers. Bracts may persist until anthesis. Sepals are regularly gland-serrate. The 7-15 stamens have pink anthers; there are 2-5 styles, averaging close to 3. The fruits are bright crimson, 11-15 mm. in diameter, conspicuously pruinose. The nutlets bear prominent dorsal ridges, and are 6-7 mm. long.

In some of these characters *C. danielsii* approaches the *Punctatae*, which are distinguished from the *Crus-Galli* chiefly by their thinner, more acute, usually pubescent leaves with straighter, more impressed veins; pubescent cymes; more numerous styles and nutlets; more persistent bracts. PALMER suggests that it may have originated as a hybrid between the two groups. Among the *Crus-Galli* of Missouri many of SARGENT's species occupy a similarly intermediate position. *C. parciflora*, *C. lawrencensis*, *C. leptophylla*, and *C. tenuispina* seem (as far as can be judged from descriptions and from herbarium specimens, with the reservations noted) to be distinguished by more nu-

merous styles and larger leaves, more often elliptic in shape. Others, such as *C. discolor*, *C. pilifera*, and *C. vallicola*, show a tendency to pubescence besides. Still others have smaller, decidedly more elliptic acute leaves, with various combinations of the other characters just mentioned; examples are *C. tantula*, *C. paradoxa*, *C. intermixta*. A fourth group seems to be marked by much broader leaves: *C. rotunda*,

TABLE 2  
LEAF INDEX  $\left(\frac{\text{LENGTH}}{\text{WIDTH}}\right)$  IN LOCAL  
RACES OF CRATAEGUS

C. CRUS-GALLI	PUNCTATAE
(47)* 2.3 ± 0.06	(24) 1.9 ± 0.02
(47)† 2.7 ± 0.05	(25) 1.8 ± 0.03
(48) 2.2 ± 0.04	(65) 1.6 ± 0.03
(37) 2.0 ± 0.03	(16) 1.8 ± 0.03
(70) 2.2 ± 0.08	(19) 2.1 ± 0.04
(69) 2.4 ± 0.06	(20) 1.6 ± 0.03
(65a) 2.1 ± 0.04	
(71) 2.0 ± 0.02	C. DANIELSII
(60) 2.2 ± 0.01	(8) 2.1 ± 0.03
(46) 2.3 ± 0.05	(8)† 2.1 ± 0.05
C. albanthera 2.0	(35) 2.2 ± 0.04
C. arduennae 2.0	(19a) 2.1 ± 0.04
C. candens 2.0	
C. parciflora 1.8	C. intermixta 1.7
C. pilifera 2.1	C. paradoxa 1.8
C. lawrencensis 1.7	C. tantula 1.7
C. vallicola 1.7	
C. discolor 1.7	C. jasperensis 1.4
	C. palmeri 1.3
	C. rotunda 1.3
	C. rubrifolia 1.7

\* Numbers in parentheses are those attached to individual plants for the purpose of recognition.

† Same plant measured in different years.

*C. jasperensis*, *C. palmeri*, *C. rubrifolia*. Whether such apparent differences in size and shape of leaves represent real genetic characters can be determined only by the development of adequate methods of measuring such variants. In spite of great variation even on one plant, it seems that an index of shape of leaves obtained by dividing length by width is a fairly constant figure in related plants. Table 2 presents such data from several plants of the local forms of *C. crus-galli*, of *C. danielsii*, and of a few of the Punctatae of the region. The

measurements were made in the field on 25 leaves per plant. Included in the table also are similar figures computed less accurately from herbarium specimens of some of SARGENT's species.

A further character which may be of some value in tracing relationships is time of anthesis. Near Columbia the Punctatae and *C. danielsii* flower during the first week in May, *C. crus-galli* about two weeks later. In general there is a tendency through the entire group for the more characteristic forms of *C. crus-galli* to be the latest to flower; there are, however, so many exceptions that no reliance can be placed on this single character alone.

Even from such imperfect and incomplete observations as those presented in the preceding discussion, it is evident that the species named have many of the characters of *C. crus-galli*, of the Punctatae, and perhaps of the Virides, in various combinations. The effect is that of a "hybrid swarm" of races rather than of individuals, each race restricted in range. No particular type seems to be selected by any environment; except for those with nearly round leaves, which inhabit the southwestern counties, all types seem to occur indiscriminately in all parts of the state. In such a situation it is obviously impossible to designate certain forms as distinct species while others are not so recognized. At the same time, such types as *C. danielsii* differ more from the characteristic forms of *C. crus-galli* than do the latter among themselves, and therefore must be differently ranked. The most convenient method perhaps is to group all these intermediate types into a number of varieties associated with the species which they most nearly resemble. *C. danielsii*, judging from the scanty pubescence (which is sometimes lacking), from the proportions and texture of leaves, and from the numbers of floral parts, seems rather more closely related to *C. crus-galli* than to the Punctatae. I propose therefore the following nomenclatural change:

*Crataegus crus-galli* L. var. *danielsii* (Palmer) comb. nov. (*C. danielsii* Palmer in Jour. Arn. Arb. 16:355-357. 1935).

An example of the dangers of reliance on herbarium specimens and of isolated observations is *C. danielsii* f. *glabra* Palmer (*l.c.*). This was based on a specimen in the herbarium of the University of Missouri. Unfortunately the group of plants from which the specimen came has been destroyed, and search has discovered no others

near by. But the real status of this "form" is shown by examination of various collections of var. *danielsii*. One of the marked plants (no. 8), as represented in the herbarium, had cymes scantily villose in 1931, glabrous in 1935; another plant (no. 35) likewise yielded pubescent specimens in 1931, while in 1936 careful examination in the field showed that some cymes were distinctly pubescent, others glabrous. The pubescence of leaves parallels that of cymes. The supposed glabrous form was doubtless one of the occasional glabrous branches of a variety characterized by a variable and deciduous pubescence.

DEPARTMENT OF BOTANY  
UNIVERSITY OF MISSOURI

## BRIEFER ARTICLE

### FURTHER OBSERVATIONS ON GONYOSTOMUM SEMEN

*Gonyostomum semen* (Ehrenb.) Diesing, a chloromonad flagellate, the general morphology of which has recently been described (3), was found to persist in the plankton of Cedar Swamp near Woods Hole, Massachusetts, during the summers of 1935 and 1936. In August 1936 it was discovered in even greater abundance in Iron Pond, a waterhole beside the railroad half a mile west of the swamp. Iron Pond is not situated in a sphagnum bog as is Cedar Swamp; but its plankton, like that of the latter, is composed mainly of flagellates.

During the summer of 1935, material from Cedar Swamp was fixed in Zenker's and Schaudinn's fluids (the latter containing 5 per cent glacial acetic acid by volume) prepared according to formulae in Lee's *Microtometist's Vade-Mecum* (1928), was impregnated with paraffin by the usual methods, sectioned at a thickness of  $6\ \mu$ , stained with Heidenhain's iron-alum haematoxylin, and mounted in balsam. Certain morphological features not known at the time of writing the 1935 paper were brought to light. The flagella, often preserved *in toto* by fixing in Zenker's fluid, were found to be of approximately equal length; their basal portions extend through the opening of the triangular cavity and are inserted in the cell membrane in the center of the base of the cavity. No blepharoplasts could be identified at the points of insertion of the flagella, but distinctly chromatic threadlike structures were seen extending from these points of insertion to the anterior end of the nucleus, where no bodies could be recognized as centrosomes. The nucleus, as studied in the stained sections, is an oval body apparently surrounded by a nuclear membrane. Inside this apparent membrane is a matrix of small closely packed chromatic granules of rather regular size which stain conspicuously with Feulgen nucleal reagents. Scattered at random through the nuclear matrix are from one to ten larger chromatic bodies (endosomes?) measuring  $1-2.5\ \mu$  in diameter. Figure 1 gives a diagrammatic representation of the nucleus and of the kinetic apparatus as we have observed them. The picture of the resting nucleus is much the same as that described by FORT (4) in *Vacuolaria viridis* (Dang.) Senn, but rather different from that indicated in the original description and figures of another chloromonad,

*Reckertia sagittifera* Conrad (2). The kinetic apparatus of *G. semen* as here represented is far less complex than that described by FOTT in *Vacuolaria viridis*.

Further reports of *Gonyostomum semen* have been made by SMITH (5), who collected it in two sphagnum pools in West Yorkshire, England, and by CHADEFAUD (1), who described small mucus-producing bodies independent of the trichocysts in material collected by M. Lefèvre in a lake in Rambouillet Forest, 50 km. southwest of Paris. M. Lefèvre, in a letter dated July 2, 1936, informs us that this lake has a pH of 6.0-6.2 and an abundant aquatic flora of vascular plants, desmids, Protococcales, flagellates, diatoms, etc.

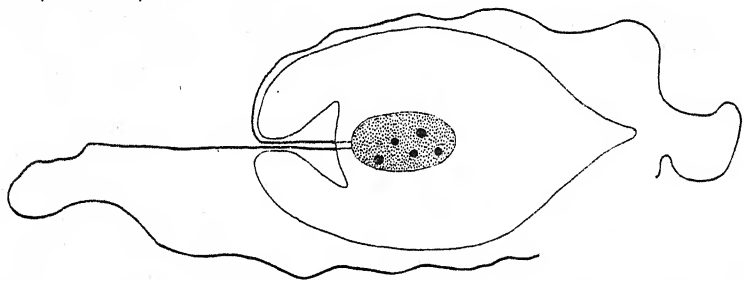


FIG. 1.—Diagrammatic dorsiventral longitudinal section of *Gonyostomum semen* from Woods Hole, Massachusetts, showing granular structure of nucleus and reconstruction of kinetic apparatus as seen in fixed and stained whole and sectioned material.

Specimens of *G. semen* taken from Cedar Swamp and from Iron Pond during 1935 and 1936 have been placed in the Herbarium of the Marine Biological Laboratory. We acknowledge gratefully the many courtesies shown to us by members of the staff of this Laboratory.—FRANCIS DROUET and AARON COHEN, *Department of Botany, Marine Biological Laboratory*.

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## CURRENT LITERATURE

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*The Cerro Cuadrado Petrified Forest.* By G. R. WIELAND. Carnegie Institution of Washington. Publ. 449. 1935. Pp. 180.

The Cerro Cuadrado petrified forest of south-central Patagonia is described in great detail by WIELAND from sections of the seed cones, fragments of stem impressions, twigs, foliage, and basal portions of two seedlings. This South American material was collected by RIGGS in 1924 and is now among the exhibited collections of the Field Museum of Natural History. Descriptions of certain North American Araucarian cones are also included and discussed.

The subject is treated in seven chapters, the first of which is concerned with a discussion of the geologic age of the deposits. Unfortunately, WIELAND accepted the opinion of WINDHAUSEN that these remains represent early Triassic deposits, although he pointed out the possibility that they might belong to a post-Triassic age. In the most recent publication of the Argentine Survey,<sup>1</sup> it is now said that the Cerro Cuadrado forest cannot be older than the Eocene. The more defined limits of the Cretaceous-Tertiary boundary have, however, been long subject to discussion.

The second chapter gives a taxonomic description of this collection of cones, represented by several dozen beautifully petrified specimens, together with other associated plant parts. WIELAND groups the cones into two generic types, *Proaraucaria* and *Pararaucaria*. Under *Proaraucaria mirabilis* he includes *Araucarites mirabilis* Spegazzini (1924), *A. windhausenii* Gothan (1925), and *Proaraucaria elongata* Wieland (1929), a series of 20 or more large spherical and ellipsoidal cones up to 9 cm. long and 6 cm. wide. They differ from living Araucarian cones in size and in the uniformly deep sulcus between the bract and seed scale. WIELAND mentions the evidence that the seeds may contain an endosperm and embryo, and this fact is established beyond doubt by a recent investigation by DARROW<sup>2</sup> on the same species.

DARROW states that the structure parallels so closely that of the scales of modern Araucarians, especially those of the *Eutacta* section, that *Proaraucaria mirabilis* furnishes no added evidence to settle the controversy centered about the status of the carpellate cone scales in *Araucaria*. However, it appears that DARROW did not show the cone scale of *P. mirabilis* in her diagrammatic sketch

<sup>1</sup> FREUQUELLI, JOAQUIN, Situación estratigraphica y edad de la zona con Araucarias al sul del curso inferior del Rio Deseado. Bol. Inform. Petrolif (Buenos Aires). No. 112. 1934.

<sup>2</sup> DARROW, BERTHA SCHWEITZER, A fossil Araucarian embryo from the Cerro Cuadrado of Patagonia. BOT. GAZ. 98:328-337. 1936.



but the condition found in a modern *Araucaria*, without the conspicuous partial separation in the fused members so clearly shown in WIELAND's figures and evident from an examination of this fossil material itself.

The stem impressions and foliage associated with these cones as well as parts of two seedlings belong to the Colymbea type of *Araucaria*, still represented in South America by the forests of *A. imbricata* found growing today on both flanks of the Andes about 600 miles farther north. A few of the *Proaraucaria* cones are treated as varieties of *P. mirabilis*. The fact that DARROW finds many Eutacta features in the cones serves to indicate that the two branches of the genus were not so sharply separated in past geologic time as they are in the living species.

*Pararaucaria patagonica* includes a collection of more cylindrical cones of smaller size, at least 5 cm. long and 2 cm. wide; somewhat larger than this if the missing eroded portions were restored. Like *Proaraucaria*, these forms are separated from living Araucarians by the much deeper separation of the cone scale and bract, and they differ from the former by the presence of important intermediate characters that would tend to unite them with *Pinus*, or place them closer to the Abietineae.

WIELAND includes the descriptions of several North American Araucarian cones, so that this monograph probably contains descriptions or photographs of all American material in which internal structure is preserved.

A chapter is devoted to the description of noted petrified forests found in all parts of the world, descriptions of log rafts and other conditions which may lead to petrification, followed by a chapter on the chemistry of petrification. Here the author brings together a discussion of some of the little known facts and theories bearing on petrification.

Chapter 5 is a treatment of cone and flower in which the inflorescence theory of the conifer seed cone and the ligular theory are contrasted and discussed. The former, sometimes spoken of as the brachyblast theory, is strongly favored as against the ligular theory. Of course there are many forms and variants of these theories which were not discussed, and WIELAND could scarcely review all that has been written in this century-old discussion of the morphology of the conifer cone within the space allotted. In his final chapter the author puts himself clearly on record as favoring the inflorescence theory. The Araucarian cone scale as shown by WIELAND's sections appears to be much more than a ligule. Unfortunately, DOAK's monograph<sup>3</sup> on pine needles and cones was still in press and could not be consulted, but it is significant that these two investigators, one working on the Araucariaceae and the other on the Pinaceae, have independently reached substantially the same conclusions. Both add many original observations on the morphology of cone development. DOAK regards the seed cones of the Pinaceae as compound structures, rep-

<sup>3</sup> DOAK, C. C., Evolution of foliar types, dwarf shoots, and cone scales of *Pinus*. Illinois Biol. Monographs 13, no. 3. 1935.

representing a long-shoot axis with the secondary branches represented by the cone scales, while the pollen cones are regarded as simple cones borne in the axillary position of the dwarf branches.

There is a beautifully illustrated chapter describing the living Araucarian forests of South America and Australia, and a discussion of the former more worldwide distribution of *Araucaria*, now limited entirely to the southern hemisphere.

Whether a further investigation of the geologic age of these Araucarian fossils will actually prove them to be Triassic or Tertiary, this contribution has significance in demonstrating that this group possessed cone scale and bract which were actually more nearly separate and distinct in past geologic history and have become fused in modern forms, so that *Araucaria* may be derived from other coniferous stocks and need not be looked upon as a separate and distinct phyletic line.—J. T. BUCHHOLZ.

*The Cultivated Races of Sorghum*. By J. D. SNOWDEN. London: Adlard & Son, 1936. Pp. 274. Illustrated. 10s. 6d.

This valuable work is the most comprehensive yet published on the taxonomy of the genus *Sorghum*. It is the result of studies of panicles in the flowering and mature stages filed in the herbarium of the Royal Botanical Gardens at Kew, England. Most of the specimens were obtained from the British possessions, but all important sorghum producing countries, including the United States, are represented.

The book includes an extensive review of previous classifications, an elaborate bibliography of the classification and history of sorghums, numerous references to their culture and physiology, and an index listing nearly all of the Latin or botanical names and more than 1600 vernacular names that have been applied to varieties. The source of each variety is indicated. Historical, agricultural, and economic notes in addition to the keys and botanical descriptions are included for many varieties and species. The book concludes with a chapter on the relationships of the wild and cultivated sorghums.

The cultivated annual sorghums, often considered as one species, *Sorghum vulgare* Pers., are here classified into 31 species, 10 of which have been created by the author. He states: "There is a very wide range and much diversity in the shape and size of the fertile spikelets and grains, the texture of the glumes, and the relative size of the glumes and grains, and their relationship to one another. . . . To sink such distinctive races of cultivated Sorghums all under one species is a decidedly retrograde step, which possesses no advantage whether from the standpoint of the practical cultivator, the geneticist, the cytologist, or the systematist . . . the recognition of the most distinct races as species has the advantages of simplicity and directness. It also facilitates the preparation of a key to the cultivated sorghums which can be reasonably clear and not necessarily complicated. This is a task which is almost impossible of satisfactory

accomplishment under any system which attempts to crowd all these exceedingly numerous cultivated varieties into one vast unrestricted species."

Regardless of the advantages mentioned by the author, the separation of a group of varieties, having the same chromosome number and showing perfect interfertility among the hybrids, into a multiplicity of botanical species would seem to be questionable procedure in the light of modern genetics and cytology. Hybrid segregates from a single cross between two widely different sorghum varieties might easily fall into several of the author's so-called species, and possibly require the creation of additional species names in order to classify them.

The 31 species are subdivided into botanical varieties (subspecies) and these in turn into numbered forms. The cultivated varieties are listed in many cases without showing any distinctions between widely different varieties within a form. As an example, several distinct varieties of black-glumed kafir are listed under a single form and the very different group, hegari, is listed under the same form with only one of the numerous distinguishing characters separating hegari from the kafirs being indicated. In another form are listed three distinct American varieties, Dawn kafir, Reed kafir, and Chiltex, and several South African varieties with no attempt to distinguish between them. The reviewer is unable to determine the extent of such incomplete or erroneous groupings owing to insufficient familiarity with varieties not grown in America.

The studies were restricted to dried panicles without the opportunity of observing characters of other parts of the plants. Notes on plant characters were received from some experimenters and collectors sending specimens, but these were not usable generally because they were lacking for many other specimens. A classification of cultivated crop varieties, based upon morphological characters of herbarium specimens alone, of necessity leads to incongruous or artificial groupings that might be avoided by consideration of the characters of growing plants. Thus sweet and juicy-stemmed sorghos of the late maturing South African varieties, Honey and White African, and of the early, drought evading Chinese variety, Chinese amber, are included in the species *Sorghum dochna*, along with the broomcorns from southern Europe which have dry, non-sweet stems and very long panicle branches. Differences in seed size, and particularly in glume or seed color resulting from differences in environment, degree of maturity, or exposure to bleaching or weathering after maturity, have caused the author to place the same or very similar varieties in different subspecies. As an example, Dawn kafir is found as *S. caffrorum* var. *albofuscum* and again as var. *albidum*.

The major characters used to separate species, including the shape and size of the sessile spikelets, the thickness and degree of opening of the glumes, and the size of seeds, are all quantitative characters that frequently are difficult to classify correctly. Such characters as the persistence of glume hairs and pedicelled spikelets are subject to wide variation in dried material because of the shedding of these parts during maturity and handling.

Despite certain discrepancies, this book is an extremely useful reference work, and obviously represents an extensive and painstaking study of an immense collection of material.—J. H. MARTIN.

*Production of Field Crops.* By T. B. HUTCHESON, T. K. WOLFE, and M. S. KIPPS. New York: McGraw-Hill Book Company, 1936. Pp. xvii+445. figs. 110.

The present revision of a text now in use for more than a dozen years brings the subject matter more nearly up to date, as nearly as can be done in a text of this type. It has been somewhat shortened by the omission of some illustrations and tables; other new material has been added.

The general discussion chapters, as well as those dealing with specific crops, furnish interesting examples of the principles of ecology applied to crop plants. The distribution maps are clear cut, most of them based on data for 1929. Most of the illustrations have real point, but it seems unfortunate that agricultural texts should continue to use illustrations both inaccurate and misleading, as exemplified here by those of the corn grain and potato tuber.—E. J. KRAUS.

*Encyclopedie Pratique du Naturaliste. XXX. Code Universel des Couleurs.* By E. SEGVY. Paris: Paul Lechevalier. 1936.

A handy pocket-size folder contains 48 cardboard loose plates with 15 tints on each. The first color on each plate is a net color derived from blue, red, or yellow, which are the basic colors used. This initial color is progressively lightened to white in the first vertical column. Each shade thus obtained is toned down in a horizontal direction by the use of black, red, blue, yellow, or its own complement. Thus 720 different colors are derived.

Comparisons with various objects may be made direct, or by using masks of the color complementary to that of the shade isolated to obliterate the other tints on any particular card while a comparison is being made. Such masks are included in the set of cards. There is also a list of colors and a convenient index.—E. J. KRAUS.

*The Biochemistry of the Lipids.* By HENRY B. BULL. John Wiley and Sons. 1937. Pp. ix+169. Illustrated. \$2.75.

This work is the successor to the mimeographed publication which appeared some months ago. The material has been in part rewritten, rearranged, and is better organized. There are nine chapters, besides the introductory one. These deal with the fatty acids; soaps; alcohols, waxes, and hydrocarbons; the sterols and related compounds; fats and oils; the phospholipids; cerebroside; carbohydrate esters of the higher fatty acids; and emulsions. Author and subject indices are provided.—C. A. SHULL.



# THE BOTANICAL GAZETTE

June 1937

## ZINC AS A NUTRIENT FOR PLANTS<sup>1</sup>

W. H. CHANDLER

(WITH FIVE FIGURES)

As early as 1869, RAULIN (43, 44) presented strong evidence that zinc is essential in the culture medium for at least some fungi. He thought it was not merely a stimulant but an essential element, and that growth when zinc is not added to the medium is due to the presence of zinc as an impurity. JAVILLIER (22, 24, 25) emphasized this point of view and showed that, besides being an impurity in the purest chemicals that could be purchased, zinc was apt to be present in glassware. When he grew *Aspergillus* in Bohemian glass or quartz without addition of zinc to the medium, there was much less growth than when he grew it in Jena glass. Other workers (14, 30-34) did not find results in harmony with the views of JAVILLIER, probably because they did not exclude as many of the sources of zinc as an impurity. This view seems justified, especially by the work of STEINBERG (50-57), in which he was able to reduce the amount of zinc as an impurity so much that growth by *Aspergillus* when zinc was not supplied was less than 10 per cent of that when it was supplied. Some workers before STEINBERG thought of zinc not as an essential element but merely as a stimulant for fungi: zinc present as impurities was enough for apparently normal growth, so that the additional growth when zinc was added seemed merely a stimulation (8).

<sup>1</sup> A summary of work that has been or is to be published in cooperation with D. R. HOAGLAND, P. L. HIBBARD, J. C. MARTIN, and P. R. STOUT. Paper presented at a Symposium on Mineral Nutrition, December 1936 Meetings of the A.A.A.S.

RAULIN (44, 45) thought zinc was an essential element for higher plants also. JAVILLIER reported work with water cultures in which minute quantities of zinc in the culture medium caused increased growth. Other workers before and after this obtained suggestions of response to zinc by higher plants in water culture and in sand culture. The careful work of MAZE (35-37) with maize plants grown in water culture, and the work of SOMMER and LIPMAN (47, 48, 49), in which great care was used to prevent the plants from obtaining a zinc supply from the glassware, the water, the dust of the greenhouse, or the chemicals in the nutrient solution, seem to establish beyond question the requirement of zinc as a nutrient by some plants if not by all. HAAS (18) reported a benefit from zinc to lemon trees grown in water cultures. More recently HOAGLAND *et al.* (20, 21) have described, from water cultures, zinc deficiency symptoms on several other plants, including young apricot trees.

There have been reports of increased growth and yield by plants in the field in response to applications of zinc to the soil, and reports of disease symptoms cured by such applications. JAVILLIER (23) apparently found 5.5 to 55 pounds of zinc sulphate per acre to increase the growth of wheat, oats, maize, lupines, and peas. Beginning in 1927, workers in Florida (5, 6, 7) reported benefits to maize, peanuts, peas, millet, and some other annual plants, from treatment with zinc. Zinc deficient maize plants showed chlorotic streaks between the veins; this disease was called white-bud.

MOWRY and CAMP (39) found that zinc sulphate, as little as 0.5 pound to the tree, applied to the soil, would cure serious injury to tung oil trees, and even 0.25 pound each caused striking improvement to mottle-leaved Satsuma orange trees in Florida.

ALBEN *et al.* (2, 3) in Louisiana, DEMAREE *et al.* (15) in Georgia, and FINCH and KINNISON (16) in Arizona have found that zinc sulphate will cure rosette of pecan trees, a disease that causes, in addition to rosettes of small leaves with shortened internodes in spring, yellow mottling between the leaf veins, later some crinkling of leaves, and sometimes dying back of the shoots.

CURING AN ORCHARD DISEASE WITH ZINC.—In 1931 and 1932, four different groups of workers discovered independently that treatment with zinc, through the soil and in other ways, will cure a serious

orchard disease, one that affects trees of some kinds in some areas and of all kinds in some areas from Florida westward through the Gulf states, Texas, Arizona, and California, and northward at least to the Canadian line. Many millions of dollars worth of land otherwise excellent for orchards are worthless unless zinc is supplied to prevent or cure this disease. On the pecan and the apple it is called rosette; on the citrus fruits, mottle-leaf; on the walnut, yellows; on grapevines, stone-fruit trees, and some other trees, little-leaf. Trees are cured, however, with zinc whose symptoms are not described by any one of these terms; and not all rosetting, mottling, or yellowing is cured with zinc. The only sure means of identifying the disease is the response of the trees. In this paper, therefore, I shall call the disease zinc deficiency, even though the evidence now available may not prove beyond question that it is due merely to lack of zinc as an essential element.

### Observations

In California (10-13), following the work of HAAS and REED (19), among the first trials were treatments with a mixture of many minor elements including 90 pounds to the acre of zinc sulphate, a larger quantity than was necessary to cause healthy growth of injured plants in Florida soils. There was no evidence of response, however, and, as in Louisiana (2, 3), the effectiveness of zinc in curing this disease in California was discovered somewhat accidentally (10) by using ferrous sulphate that contained a considerable amount of zinc. Since this discovery in 1931, plants have been treated continuously in more than fifty districts over an area more than 400 miles long in the San Joaquin, Sacramento, and other valleys. Trees have been treated with zinc sulphate through the soil, through holes in the trunk, by driving pieces of zinc or galvanized iron into the trunk or branches, by spraying the foliage with zinc sulphate and lime or with zinc oxide, and by spraying when the foliage was off with zinc sulphate or zinc chloride solution.

Soil treatments tend to be more effective the nearer the zinc compound is placed to the trunk of the tree. In the soil with the lowest fixing power for zinc of any studied in this investigation, 1000 pounds of zinc sulphate to the acre spread over all the soil would cure nearly



all the trees and keep them healthy for about three years; 300 pounds to the acre all within 2 feet of the trunks was more uniformly effective and the benefit lasted as long; in some soils, 2500 pounds to the acre all within 2 feet of the trunk was not so uniformly effective and the benefit never lasted so long. Adding with the zinc sulphate one to three times as much ferrous sulphate, aluminum sulphate, or calcium sulphate, slightly reduced the amount necessary to cure a tree but did not prolong the time before symptoms would show again. Mixing ammonium sulphate with the zinc sulphate also increased its effectiveness slightly, but mixing complete fertilizer (17) with it seemed to reduce its effectiveness a little.

Zinc sulphate powder packed into holes around the trunk about 3 inches apart, about 2-3 gm. to the hole, and covered with a wax that is harmless to the tissue, has never failed to cure a tree that showed zinc deficiency. The benefit usually lasts at least three years, to trees in soil with a high or a low fixing power. A considerable amount of sapwood is killed in the trunk, however, and some of the holes may remain open and permit the entrance of heart rots.

Driving pieces of zinc or of galvanized iron or zinc coated nails deep enough into the trunk or branches to hold well, 15 to 20 pieces nearly an inch wide or 50 to 60 zinc coated nails for each inch of trunk diameter, will cure zinc deficient trees of all the kinds tested except citrus. On stone fruits and the walnut the pieces should be kept an inch apart or there may be killing of the bark and cambium between them. The benefit seems to last longer than that from any other method of treatment, although it has been tried for only four years. This seems to be the most satisfactory method of treating walnut trees and cane-pruned grapevines. Pieces driven into one branch will cure the parts of that branch above the pieces but not the parts below them.

Spraying the foliage with zinc sulphate, 10 pounds or somewhat less with half as much hydrated lime in 100 gallons of water, has been found surprisingly effective for citrus trees (27-29, 40-42). It is moderately effective for apricot trees and grapevines, but almost completely ineffective for other kinds of deciduous trees tested (12, 13). For citrus, zinc oxide seems a little more effective than

zinc sulphate and lime, and it can be combined with other sprays, including oil sprays.

Spraying in winter (13), after all leaves are off and before the buds begin to swell, with 25 to 50 pounds of zinc sulphate, considerably less of zinc chloride, in 100 gallons of water, has cured apple trees and stone-fruit trees but has failed to cure walnut trees (fig. 1).



FIG. 1.—Walnut tree showing zinc deficiency symptoms. There are no rosettes, but the leaves are rather small, mottled, and crinkled.

Zinc chloride may have benefited walnut trees somewhat. Spraying must be repeated each year, at least during the first four or five years after treatment of badly affected trees begins.

In soil treatment, fixing power for zinc determines the amount that must be applied for a cure. In treatments above ground, fixing power for zinc and degree of zinc deficiency have little to do with responses, except that in dormant spraying stronger solutions are necessary in the first and sometimes in the second year of spraying, if the shoots have been killed back badly by lack of zinc.

Following treatment during late summer, autumn, or winter, in the soil, in holes in the trunk, or by driving zinc into the trunk or branches, the rosettes of small leaves show in the first spring; improvement begins to show two or three months after growth starts. Following winter spraying, the leaves show healthy from the beginning of spring growth: buds that without the spraying would open into rosettes of small, mottled leaves expand after the spraying into shoots bearing normal leaves.

The best treatment for grapevines pruned to spurs with two or three buds seems to be to spray or swab them immediately after pruning with 2 pounds of zinc sulphate to a gallon of water; spraying unpruned vines or vines pruned late enough in winter to bleed, even with such strong solutions, is not effective. On trees, pruning wounds are not so helpful toward getting zinc into the wood.

#### SYMPTOMS OF ZINC DEFICIENCY AND SUSCEPTIBILITY OF DIFFERENT SPECIES

By use of all these methods, one or more of which have been successful on all species, it has been possible to determine with reasonable certainty what symptoms may disclose zinc deficiency for the different orchard species under different conditions (fig. 2). The most dependable symptoms on deciduous trees are the rosettes of small, stiff, nearly sessile leaves in the first flush of growth in spring. These leaves may or may not be mottled and they never become more than 1 inch long or about 0.25 inch wide. From below them, further below on apple trees than on stone-fruit trees, shoots tend to start that may bear healthy green leaves throughout the summer, the appearance of the trees in late summer often being better than that of healthy trees, because they have not been bedraggled with fruit. Trees affected more severely may bear leaves on these later shoots more or less mottled from the beginning, with badly mottled, small, and abnormally shaped leaves toward the end of the season's growth. These same symptoms were observed by HOAGLAND *et al.* (20) on apricot trees grown in zinc deficient water culture. Some trees begin to die back when they have been affected a year or two, others after many years. Spring rosettes of small leaves are rare on cherry trees and Persian walnut trees, and are not

always shown by zinc deficient citrus trees. In fact, old, unpruned trees of all kinds rarely show typical rosettes of small leaves in



FIG. 2.—Zinc deficient apple tree photographed in July, showing rosettes of small leaves and little other growth. A tree mildly affected would have a considerable number of normal shoots starting from below the rosettes.

spring except on suckers. In orchards of kinds such as the almond, that are pruned lightly so that the shoot growth is very short, it is

often impossible to distinguish the symptoms of zinc deficiency from those of other influences, such as nitrogen deficiency, crown gall, or nematodes, that cause leaves to be small.

If any fruit sets on badly affected branches, it is mostly worthless and shows symptoms characteristic of the species. Peaches and Japanese plums, for example, are smaller, more flattened, and more beaked than normal ones; apricots are small and less flattened (fig. 3); lemons are small, oblong, and pointed. There is also a tendency for stone fruits to have brown areas in the flesh and to break down early. Walnut trees bear small poorly filled nuts, with tough, somewhat pliable shells instead of the normal brittle ones.

There is always a reduction in the number of fruit buds on trees rather badly affected, but stone-fruit trees usually have enough flowers for a fair crop if a good percentage of them set fruit. Usually, however, nearly all the flowers fail to set on the badly affected parts of the tree. Often the upper part of the tree is badly affected and bears only a few abnormally shaped fruits, while the lower part bears normal foliage and at least a moderate crop of fruits nearly all of which are approximately normal in appearance. A winter treatment with zinc that causes the first foliage in spring to be normal also causes the flowers, if there are any, to set fruit and causes the fruit to be normal in size and form.

All orchard and ornamental trees and shrubs grown in the valleys of California seem to show injury if the zinc deficiency is great enough, but the differences in susceptibility are large. For example, peach trees may be dying from insufficient zinc in a soil on which Oriental plane trees and grapevines of some varieties are healthy, but the situation is so severe in some soils that plane trees and grapevines are seriously injured unless they are treated with zinc. The most susceptible seems to be the sweet cherry tree, which may be dying badly from insufficient zinc in soils on which trees of all other kinds are healthy. Then following in order seem to be the apple, the Japanese plum, possibly the *Domestica* plum, the peach, the walnut, the apricot, the avocado, citrus fruits, some varieties of grapes, and the fig. This may not be the true order in all cases. I cannot place, from experience, the pear or the pecan, but from limited observation they seem more susceptible than the walnut.

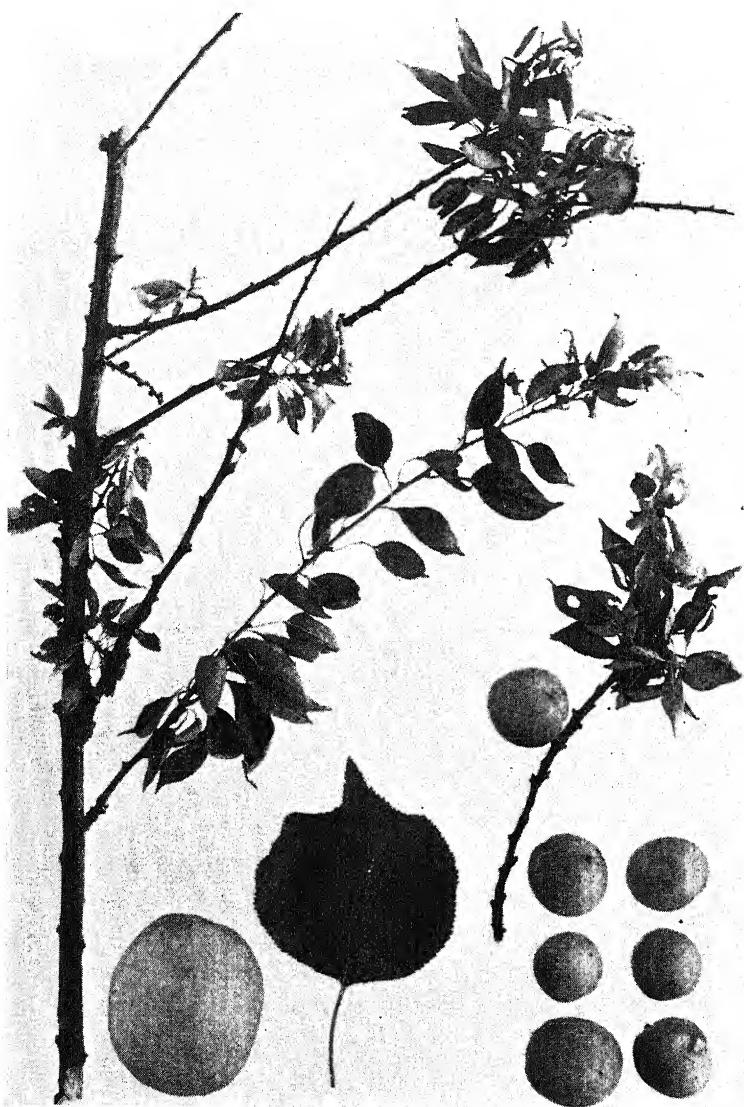


FIG. 3.—Abnormally small leaves and fruit from zinc deficient apricot tree. The fruit and leaf at lower left are from a tree which does not show symptoms of zinc deficiency.

## ZINC DEFICIENCY IN CORRAL AREAS

In California, soils in which trees are most apt to show zinc deficiency are usually sandy or gravelly. Trees on compact clayey soils rarely show it except where there have been old corrals or where there has been heavy manuring for hotbeds or in truck growing, and where there have been Indian camps. In such areas trees tend to show rosettes of small leaves and other symptoms of zinc deficiency younger than even in the sandy soils. A few groups of trees on such areas have been completely cured with zinc, but they had been sprayed with Bordeaux mixture. Trees in other such areas have made striking response to zinc but have shown other symptoms than those of zinc deficiency. Copper and manganese in addition to zinc are being tried this year.

Four annual applications of 60 tons of fresh horse and cow stable manure to the acre or the equivalent amount of potassium, of phosphorus, or of nitrogen, have not yet produced symptoms of zinc deficiency on healthy trees or grapevines in soils that require old corral or hotbed influences to induce these symptoms. Perhaps time is required for such influences to develop in the soil. The element found in greatest excess in corral soils, and apparently in Indian camp soils, is potassium. Bringing the content of potassium to well above that of corral or Indian camp soils by application of 4 tons to the acre of potassium carbonate has not injured the trees in the least.

WHY SOILS SOMETIMES FAIL TO SUPPLY ZINC  
ENOUGH FOR TREES

In the soil studied most, peach trees made exceptional growth during the first five years and bore 14 tons of fruit to the acre in their fifth summer. In that year zinc deficiency symptoms began to show in the tops in late summer. In three more years many of the trees were dying. New trees planted in the position from which these were pulled would show zinc deficiency symptoms in their first summer in the orchard and never made much growth unless they were treated with zinc. Analyses by HIBBARD indicate that there are more than 3000 pounds of zinc to the acre in this soil within the root zone, and that at seven years of age the orchard had removed little if any more than a half pound to the acre in roots, top, and

fruit. At first these trees had obtained zinc enough for excellent growth and had removed only an insignificant part of the zinc supply in the soil. Why could they not obtain enough zinc after four years and why could not young trees following them obtain enough? Manure in a corral must add some zinc to the soil. Why does it reduce the supply obtained by the tree? Alfalfa growing among the trees will prevent zinc deficiency symptoms (38) in many western orchards and reduce the severity in all. It seems to increase the amount of zinc taken in by the tree. How does alfalfa exert this influence?

ALBEN and BOGGS (1), working in Texas and Louisiana, seemed to find that rosette, when found on pecan trees growing in an acid soil, could be explained by a very low total zinc content, about 116 to 136 pounds to the acre in the root zone, and that when found on trees in a basic soil with a much larger quantity of zinc it could be explained by the insolubility of zinc at such reactions, pH 7.4 to pH 8.5.

In California, trees on rather clayey soils with a reaction of pH 8.0 to pH 9.0 rarely show zinc deficiency, except on corral areas; most of the moderately basic soils on which trees do show zinc deficiency symptoms have high fixing power for zinc added to them in soluble form in the laboratory, but so also have some neutral and acid soils. In a district where the soil has the lowest fixing power for zinc of any so far studied, the reaction is usually pH 7.0 to pH 7.3, and nearly all stone-fruit trees show pronounced deficiency symptoms and will die young if zinc is not applied to them. Bringing the reaction down to pH 6.2 at the surface and pH 6.5 at a depth of 4 feet by application of sulphuric acid did not improve zinc deficient trees growing in this soil. One soil was found to have a very high fixing power for zinc and has an acid reaction, pH 5.9 to pH 6.4. In this soil 1500 pounds of zinc sulphate to the acre spread on the surface was rendered unavailable so quickly that apple trees were never cured completely by it, although less than 75 gm. of zinc sulphate in holes in the trunks cured large trees in the same orchard and has kept them healthy during more than four years, without retreatment. It may be that much of the zinc in this soil and of that applied as zinc sulphate is fixed only in replaceable combinations, as a considerable amount of the zinc applied by JONES, GALL, and



BARNETTE (26) to acid soils was, and that something else prevented the trees from taking it out of this fairly easily available combination fast enough for their needs. In fact it seems probable that in all these sandy or sandy loam soils, as well as in corral areas, in the Pacific slope where zinc deficiency causes so much loss, the explanation must be sought in some influence outside of purely inorganic chemistry. If the reaction of the soil and its total zinc content determined the supply to the growing points of the plant, then it would be expected that annual or herbaceous plants would show zinc deficiency where trees do. Yet many of the soils on which trees die young unless they are treated with zinc are exceptionally good for annual plants and alfalfa, without the application of any zinc (fig. 4).

In HOAGLAND'S (20, 21) studies, in the cool summers of Berkeley with their mild sunlight, neither fruit trees nor annual plants have tended to show zinc deficiency symptoms when grown in soil taken from near the surface of any of the areas where the disease is severe in the orchard. However, when the soil was taken from 3 to 5 feet deep in some of the areas and the plants were grown during the summer months in a greenhouse, or outside but protected from cool ocean winds, young apricot trees and many annual plants, including sunflowers, showed zinc deficiency symptoms. Maize showed typical white-bud. The symptoms were corrected by addition of zinc to the soil. Out of doors in the cool ocean wind the symptoms have not yet developed, and they develop slowly, if at all, during the winter months in the greenhouse. It seems that temperature and the amount of daylight may have something to do with development of these symptoms.

ROOT INJURY IN ZINC DEFICIENT ORCHARDS.—Many facts suggest that something in these soils prevents the roots either from absorbing available zinc or from transporting it to the tops. In these injured orchards there is some dying of the roots on trees that have been badly affected for a number of years, so badly affected that weak foliage might explain the root injury. However, trees affected only badly enough to prevent fruiting, not badly enough to prevent them from holding a considerable leaf surface throughout the summer, showed no injury to the roots that could be detected by field examination in deep trenches. Potassium seems to be leached out of the

bark of the root of zinc deficient trees until it contains only 10 to 25 per cent as much potassium as the bark above ground or as the bark on roots of healthy trees or of trees cured by zinc treatment. This fact, together with the fact that something besides the reaction



FIG. 4.—Excellent growth of sunflowers in a soil on which peach trees will begin to die back in the first or second year in the orchard, if not treated with zinc.

of the soil and its supply of zinc influences the amount of zinc the top receives, suggested that some unfavorable condition in the soil renders the roots incapable of absorbing or of transporting zinc normally. Because zinc enters readily into organic combinations, it is possible that the condition in the roots which permits the leaching out of potassium might favor the combining with and holding of zinc by the roots. A few analyses suggested that zinc may accumu-

late in the roots. More extensive study, however, failed to support this suggestion.

Furthermore, experience in treating trees above ground, by spraying or by driving pieces of zinc into them, shows that without any change in the soil or in the supply of zinc to the roots of zinc deficient trees, these roots are capable of supporting at once a strong growth in the top. For example, in March, 1934, E. R. PARKER sprayed some grapefruit trees with zinc. The crop on the unsprayed trees was 56 pounds each; that on the sprayed trees, from blossoms that opened only a few weeks after the spraying, 477 pounds each, and the individual fruits were 66 per cent larger, all expressive of the greatly increased and healthy foliage. In one whole orchard, about 30 acres of mature Navel orange trees, the crop harvested the first autumn after a January spraying with zinc was said by the owner to be six times as large as the orchard had ever yielded before. About as striking results have followed dormant spraying of deciduous fruit trees with zinc sulphate (fig. 5). The strong healthy shoot growth follows from the first opening of buds in spring, instead of waiting upon improvement in root growth. There is little or no movement downward from points of treatment above ground. For example, when a branch is treated by driving pieces of zinc above some of its lateral branchlets, only the part above the zinc is cured. Branchlets below the zinc pieces still show zinc deficiency four years after those above the pieces have begun to show healthy growth. It seems certain that if much zinc had been moving downward from these pieces some of it would, within four years, have moved outward to these lower branchlets and have cured them. It seems that improvement in root growth after a treatment with zinc above ground is caused by improvement in foliage. In other words, for resumption of healthy growth and capable absorption, the roots require no other change than the substitution of healthy foliage for the weak, zinc deficient foliage.

SOIL ORGANISMS IN ZINC DEFICIENT ORCHARDS.—The soil flora seems to have some part in bringing on zinc deficiency. ARK (4) found that sterilization of the soil with steam, formalin, or ether caused plants to make as good growth as they made when treated with zinc sulphate. HOAGLAND (21) obtained similar results with



FIG. 5.—Cherry tree showing pronounced zinc deficiency symptoms. Most of the branches have died. The large leaves are on a shoot that had pieces of zinc driven into it early in the spring.

maize in this soil. After he sterilized the soil by autoclaving or with formalin, growth was as good as when he applied zinc sulphate to it. ARK (4) has isolated from soils on which trees showed pronounced zinc deficiency two strains of bacteria that, growing in the culture medium, cause maize, young apricot seedlings, and young walnut seedlings to show striking symptoms that are corrected by injecting zinc into the stems.

**THEORIES TO EXPLAIN ZINC DEFICIENCY.**—If all the symptoms that are cured with zinc are due merely to deficiency of zinc as an essential element, what causes the deficiency? All the facts presented might possibly be explained if there entered into the soil from the tree roots, or from manure in old corral areas, substances favorable to the ascendancy in the soil flora of species that absorb zinc in exceptional quantities, and if these species growing close to the root should take the zinc dissolved by acidity from the roots. Improved growth of plants in soils after sterilization might then be due partly to the fact that these organisms are no longer absorbing zinc and partly to the zinc supply released from them when they are disorganized. If the much larger supplies of nitrate nitrogen can be depleted by organisms when the supply of suitable carbon compounds in the soil is abundant, it does not seem unreasonable to suggest that, under conditions favorable to the selective growth of organisms that fix zinc, the minute quantities of zinc made available in the soil at the root surfaces may be so depleted.

The fact that zinc enters into many organic combinations suggests an alternative theory. The observations reported might possibly be explained if organisms such as those isolated by ARK excreted (or upon dying, yielded) substances that combined with zinc and held it insoluble. ARK, with ether and with alcohol, has extracted from a soil in which trees showed pronounced zinc deficiency symptoms, material that, poured into a culture medium, caused maize to show white-bud. He cured these symptoms by injecting zinc into the stem above the culture medium. It does not seem impossible that the heat of sterilization, the formalin, or the ether could break up such combinations and release the zinc.

I do not suggest a specific answer to the question as to how alfalfa among the trees enables them to obtain slightly more zinc, but it does not seem impossible that alfalfa roots could, by their acidity,

bring some zinc into solution, or by some influence on the soil flora, reduce the amount of zinc taken from the tree roots.

Some data seem difficult to harmonize with the view that all benefits from treatment with zinc are merely the result of supplying a deficiency. For example, in the second or third crop in the greenhouse on the soil HOAGLAND used, maize plants supplied with no zinc tend to make less growth than they should make from the zinc in the seed, less than they do make in special zinc free distilled water, yet plants treated with enough zinc sulphate will make normal growth. It might be held from such evidence as this that, both in water culture and in soil culture, zinc merely corrects something, perhaps some toxic substance from the microflora of the nutrient solution and of the soil. Much of what we are learning about responses of plants to zinc, however, does not seem to be explained by such a theory. The response of *Aspergillus* was in a sterilized medium. The responses of tree parts to treatment far above ground could be explained by such a theory only if the toxic substance were denatured when it reached the zinc after moving through the part below without injuring it. The injury in zinc deficient trees in spring and in late summer when symptoms are most striking is greatest in the uppermost parts. A toxic substance, therefore, would have to pass the fully open leaves and become concentrated enough to cause injury only in those just beginning to unfold. Other injurious substances, such as excess quantities of zinc or nitrogen compounds, often injure the lower older leaves, even cause them to fall, without injuring the young opening leaves. On the other hand, if the disease were due to lack of an essential element, it might be expected that the greatest injury would be in the top where the most growth is taking place. These facts, and many others, form a strong chain of evidence for zinc as an essential element and against the possibility of its merely denaturing toxic substances. Inconsistencies may be explained when we know more about the relation of the soil microflora to zinc and when we know more about the role of zinc in plants.

#### ROLE OF ZINC IN THE PLANT

There are 2000 to 3000 times as many nitrogen atoms as zinc atoms in tree tissue. Perhaps the proportion in other plants is simi-

lar. Workers have, therefore, generally assumed that zinc is not a permanent constituent of the compounds that form large parts of the protoplasm. The symptoms of zinc deficiency are not merely reduced growth without other striking injury, like those of nitrogen, phosphorus, and sulphur (58, 59), known to enter largely into the composition of important parts of the protoplasm; instead zinc deficiency seems to cause reduced growth only indirectly, by the injury it causes to foliage and young stems. Workers have generally suggested that it may catalyze some essential reaction. REED and DUFRENOY (46) think it may catalyze some part of the oxidation process in plants. The abundant growth that may accompany zinc deficiency injury, and the serious injury that precedes any considerable reduction in growth by such deficiency, would seem to suggest that zinc does not merely catalyze the release of energy for plant growth but may determine the direction of some process, such as oxidation, that without the influence of enough zinc may form injurious by-products.

If zinc should catalyze some process that is influenced by the carbohydrate supply, and more zinc were necessary with accumulation of carbohydrates, some of the behavior of zinc deficient plants might be explained; for example: the more severe injury on greenhouse plants in summer than during the short days in winter, the more severe injury on deciduous trees in spring and late summer when carbohydrates tend to be most abundant, the tendency of deciduous trees to improve in May and early June when carbohydrates are depleted by growth, and the more severe injury on Navel orange trees in the spring growth cycle following the accumulation of carbohydrates in winter (9).

If the role of zinc is that of a catalyzing agent, then it must catalyze some reaction that in the plant cannot be catalyzed by cadmium, mercury, silver, nickel, iron, cobalt, copper, manganese, chromium, boron, or titanium. When compounds of these elements (12, 13) were applied to branches of zinc deficient trees, through holes in the branches or otherwise, they failed to cause any improvement. There is conclusive evidence that they reached the buds. Compounds of tin, zirconium, tungsten, and molybdenum also caused no improvement; but since they produced no injury to show

that they had reached the twigs, it cannot be said with certainty that they were not all fixed in the wood before reaching the growing points.

### Summary

1. There is strong evidence that zinc is an essential element for fungi and for higher plants; but, because of the very small amount of zinc required and its widespread presence as impurities, it has required exceptional methods to hold the zinc supply to plants in water cultures low enough to prevent moderate growth. Earlier workers were able to cause some improvement by supplying zinc, but some of them considered this response merely a stimulation, because the growth of check plants was approximately normal.

2. Widespread and serious injury to trees in orchards has been overcome by treating the trees with zinc, through the soil, by driving zinc or galvanized iron into the trunk or branches, and by spraying with zinc compounds on the foliage or on the dormant twigs.

3. The trees seem to take only about one ounce of zinc a year from an acre of soil, but under some conditions they are not able to obtain enough even for this small requirement. This is not always due to a small total zinc supply in the soil or to a reaction unfavorable to the solution of zinc. The soil flora seems to be involved: some soils in which plants show zinc deficiency will supply enough zinc after they have been sterilized. Theories are suggested to explain these phenomena.

4. The role of zinc in the plants is not known, but it is rather generally thought to act as a catalytic agent in some essential reaction.

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RELATION OF NUTRITION OF TOMATO TO DISPOSITION TO INFECTIVITY AND VIRULENCE  
OF *FUSARIUM LYCOPERSICI*

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 478

WILLIAM S. COOK

(WITH SEVEN FIGURES)

Introduction

The object of this investigation was to study the nutrition of the tomato as a factor in its disposition to infection by *Fusarium lycopersici*. The nutritional plane was varied through the supply of nitrates to the cultures kept under otherwise identical conditions. As the work progressed, a study of the browning of vascular bundles as the criterion of infection became essential.

Several investigators have studied the influence of fertilizers, or of some ingredients in the fertilizers, on infection by *F. lycopersici*, and the role of nutrition under controlled conditions of the substrate.

EDGERTON and MORELAND (5) found that the addition of large amounts of lime to the soil tended to hinder the rate of development of tomato wilt.

CLAYTON (3) found that plants grown in saturated soil were markedly different in their nitrogen relations from those grown in non-saturated soil, as indicated by microchemical analysis. Resistance seemed to be directly correlated with the absence of nitrate nitrogen in the soils. He tested this relationship by growing plants in sand cultures and adding nutrient solutions. Part of the plants received a complete nutrient solution and the remainder a solution lacking nitrates. Temperature conditions in the greenhouse did not permit a virulent development of the disease, but "it was conclusively shown that plants grown without nitrate, the tissues of which plants give no nitrate test, are not infected by the fungus. Plants grown with a complete nutrient solution were readily infected" (p. 142).

AHMET (1) studied the influence of a varied mineral nutrient sup-

ply upon the pathogenicity of *Fusarium lycopersici*. His experiments were carried out under greenhouse conditions in sand cultures. Because he obtained 100 per cent infection, clear differences were apparent only in incubation time. The potassium deficient plants showed the shortest incubation time and the earliest appearance of wilt (17 days), with death of the plants in 25 days. Phosphorus deficient plants were next, with 22 and 30 day periods respectively. Excess nitrogen plants followed with an incubation period of 26 days, and with death of the plants in 37 days. Next in order were plants given excess phosphorus and excess potassium. Last were the nitrogen deficient plants, which died at the end of 58 days, even though they did not wilt.

FISHER (6) grew plants of the Bonnie Best (susceptible) and Marglobe (resistant) varieties in sand culture under deficient and excessive amounts of the essential elements. Boron and nitrogen deficiencies inhibited fungous infection in the Bonnie Best variety. Its susceptibility was decreased when the plants were treated with excesses of calcium, magnesium, and phosphorus, and solutions lacking in magnesium and phosphorus. The resistance of the Marglobe variety was decreased when the plants were treated with solutions lacking calcium and potassium and excessive in potassium, nitrogen, and sulphur.

EDGERTON (4) studied the development of tomato wilt in the susceptible Acme and the resistant Louisiana Wilt Resistant varieties in pot cultures. Different soils were used, including river sand, garden soil (loess), and two mixtures, one of soil sand and leaf mold and another of soil and leaf mold. The various soils were sterilized, heavily inoculated with *F. lycopersici*, and seeds of the two varieties planted therein. Infection results were based both on the number of plants dead from wilt and on those plants showing signs of the disease. In river sand, Acme gave 20.0 per cent infection while Louisiana Wilt Resistant gave only 3.1 per cent infection. In soil made up of a mixture of sand and leaf mold, the susceptible variety gave 92.9 per cent infection while the resistant variety gave 72.2 per cent.

EDGERTON and MORELAND (5) recognized a progressive yellowing and necrosis of leaves up the stem, leading finally to the wilted condition. CLAYTON (2) recognized a progressive wilting of the leaves,

often accompanied or preceded by a yellowing of the affected leaves. In well developed plants three to four weeks old, the first external evidence of the disease was always wilting of a lower leaf. Under conditions which were not favorable for the maximum development of the disease (resistant plants, fungus lacking in virulence, or environmental conditions unfavorable to growth of either parasite or host), there was a tendency for the disease to appear as a slow blight rather than as a wilt. In this blight the leaves yellowed and died slowly. AHMET found that young infections led, according to the conditions and degree of infections, either to a chronic disease course or to an acute one. Infection of grown plants led mostly to the acute disease course. He also recognized that death of the infected plants may come about without true wilting. He recognized yellowing and curling of leaves, stunting of plants, and bleaching of stems as additional *Fusarium* symptoms. FISHER gives a new, not previously reported, external disease symptom to tomato wilt. He states: "Adventitious roots occur on the stem which keep step with the yellowing of the leaves as the fungus spreads up the stem."

#### Material and procedure

FUNGUS CULTURES.—Cultures of *Fusarium lycopersici* used in the experiments were obtained from Dr. G. K. K. LINK of the Department of Botany, University of Chicago, and from Dr. F. L. WELLMAN of the United States Department of Agriculture. The culture furnished by Dr. LINK was used for the first experiment, but as it was thought that this culture was lacking somewhat in virulence, the latter culture was obtained and used in all of the other experiments. In pot tests made in soil with these two cultures side by side, it was found that the second culture was somewhat more virulent, although the first gave good results.

NUTRIENT SOLUTIONS.—The composition of the nutrient solutions used is given in table 1, which gives the partial volume molecular concentrations of the salts.

CULTURE OF PLANTS.—The investigation was conducted in the greenhouses of the University of Chicago. Seeds of Bonnie Best (susceptible) and Marglobe (resistant) were germinated in rich black potting soil. When they were about 5 cm. high the seedlings

were transplanted into flats containing soil, about 100 to the flat. When the plants were 7.5-12.0 cm. in height, the most vigorous were removed and transplanted into soil in 10 cm. clay pots, one plant per pot, where they were allowed to develop until ready for use. The time from planting of seed until satisfactory plants were obtained varied, being about six weeks to two months in the spring and summer and at least three months in the winter. When cuttings were used, the plants were trimmed to two leaves per plant, cut at the base just above the soil line (see the various experiments for deviations), immediately placed in buckets of water, and transplanted into the sand culture pots, two plants per pot. After transplanting, they were protected by newspapers from the sun for several days.

TABLE 1  
COMPOSITION OF NUTRIENT SOLUTIONS

SOLUTION	Ca (NO <sub>3</sub> ) <sub>2</sub>	CaCl <sub>2</sub>	KH <sub>2</sub> PO <sub>4</sub>	MgSO <sub>4</sub>
Plus nitrate.....	0.0090	.....	0.0045	0.0045
Minus nitrate.....	.....	0.0068	0.0045	0.0045

All of the experiments were carried out in sand cultures under controlled conditions, using soil-nutrient-temperature tanks (8). The aim was to grow the plants under environmental conditions most conducive to the type of growth desired. For the sand cultures, screened and sterilized white quartz sand was used; it was not subjected to any particular treatment of washing before use. An attempt was made to keep the sand below pH 6.0, and preferably at pH 5.5. Under such conditions the various salts are assimilated and the fungus grows readily. To attain this, all applications to the sand, either in the form of daily waterings with tap water or of nutrient solutions, were adjusted to pH 4.2.

Plants grown under plus nitrate culture received approximately 2.2 liters of plus nitrate solution per week, while those grown under minus nitrate culture received about 1.4 liters of minus nitrate solution during the same period. It was also necessary to flush with acidulated tap water once weekly in order to keep the concentration

of the salts in the sand below a possible toxic level. This aided in maintaining the desired acidity of the sand.

In all the experiments the temperature of the sand cultures was kept at 28° C. CLAYTON (2) found that this was the optimum temperature for growth of *Fusarium lycopersici*, and also very favorable for growth of the tomato. While he found that the air temperature was important in the development of the disease, the disease developing best under a soil temperature of 27° C. and an air temperature of 27° C., or equally as well under a soil temperature of 27° C. and an air temperature of 33° C., no attempt was made to control the air temperature in the greenhouses. The experiments extended through more than a year, with attendant variations in air temperature. Whenever possible air temperatures were kept at 21° C. during the night and at 25° C. during the day. Air temperatures up to 50° C. were recorded on some days.

INOCULATION OF PLANTS.—The ordinary plus nitrate nutrient solution in the several experiments served as an excellent growth medium for the *Fusarium* organism if supplemented with 3.3 per cent sucrose. In this solution an abundant growth was made in eight to ten days at ordinary room temperature. For cultural purposes, 250 cc. of the nutrient medium was placed in 500 cc. Erlenmeyer flasks, autoclaved, and later inoculated with a small amount of mycelium from a young culture of *F. lycopersici*.

The inoculum was either applied directly to the roots of the plants growing in sand (plants thus inoculated will be referred to hereafter as post-inoculated), or the sterile sand was inoculated previous to planting (plants referred to hereafter as pre-inoculated). In the former method the sand was pulled back from around the roots to the depth of 10–12 cm. and the contents of one culture flask (250 cc.) poured over the roots. In the latter method about 20 cm. of the sterilized sand was removed from the soil containers, the contents of a single culture poured in, and the removed sand replaced and mixed thoroughly with the inoculum. At various times during the experiments, isolations were made from the sand cultures to check the presence of the fungus. Results of these isolations showed that the organism grew well in the sand without the addition of carbohydrates.



SEEDLING TESTS.—Germination of seed and growth of seedlings took place under plus and minus nitrate nutrition. After germination, the seedlings were thinned to a definite number per pot. Infection results are based on the recovery of the fungus from any part of the stems of the seedlings. All isolations which showed fungous growth were checked microscopically.

CRITERIA OF INFECTION.—The first experiment revealed that neither the acute nor the chronic disease picture described by AHMET is an adequate diagnostic test of invasion by *Fusarium lycopersici* and of its establishment in the tomato plant. Microscopic examination of stems and plate cultures also showed that vascular browning of the stems alone, as found by FISHER, is not an invariant test. Consequently recovery of the fungus from the base of the stems, 3–5 cm. above the sand line, was used as a criterion of infection. All isolations which showed fungous growth were studied microscopically to ensure identification of *F. lycopersici*. In many cases isolations were made from the middle and top of the stem.

### Experimental results

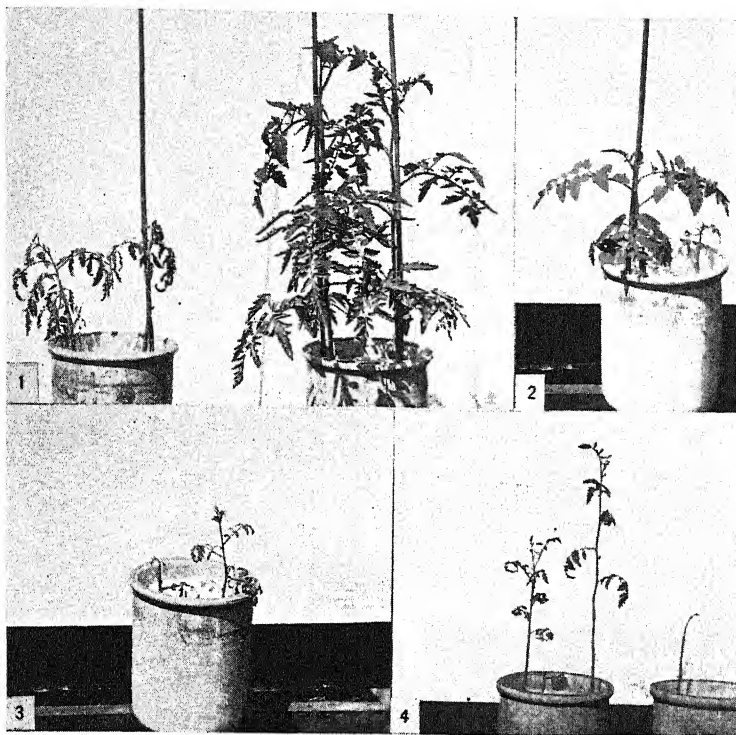
#### SYMPTOMS OF THE DISEASE

It was found that under the experimental conditions imposed, wilting was not the most characteristic symptom of tomato plants invaded by *F. lycopersici*, and that it may be completely absent in plants that die as a result of a slow necrosis of parts.

As pointed out by AHMET, two general disease pictures may be recognized: (a) the "chronic" type (figs. 1–4) in which the disease course is slow, and (b) the "acute" type (fig. 5) in which the disease course is fairly rapid, with true wilting.

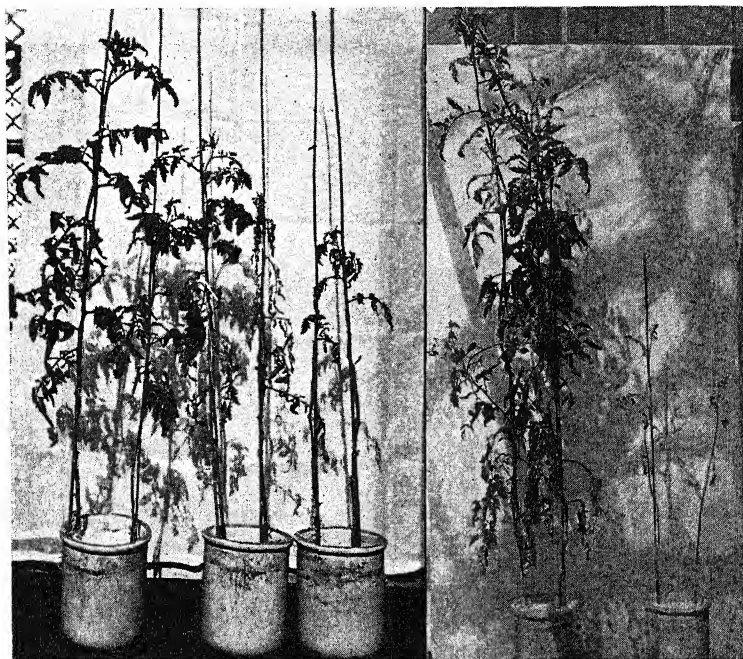
Disease symptoms appearing in the plus nitrate plants were of both the chronic and the acute type, and were abundant in the susceptible plants. Resistant plants as a whole developed few symptoms, these being of the chronic type (fig. 2). True wilt was not observed in the resistant plants.

Infected minus nitrate (high carbohydrate) plants were characterized by the chronic type of disease picture (figs. 3, 4). Symptoms were harder to follow, however, owing to the fact that yellowing and abscission of leaves was constantly taking place in the uninoculated



FIGS. 1-4.—Fig. 1, susceptible Bonnie Best, plus nitrate plants, from experiment IV. Two plants with chronic type of disease shown in culture pot on left. Note absence of lower leaves in righthand plant, also stunted condition of both plants. Control plants in pot at right. Plants under nutritional treatment since April 13, 1936. Photographed May 22. Fig. 2, resistant Marglobe variety under plus nitrate nutrition. Plant at right manifests chronic type of disease and is extremely stunted; lefthand plant apparently healthy but gave, at end of experiment (July 2, 1936), fungus on culturing. Experiment, date of photograph, and cultural conditions same as for fig. 1. Fig. 3, Bonnie Best variety under minus nitrate culture; experiment IV. Plant at left shows chronic type of disease and is stunted; plant at right apparently healthy but gave positive recovery of fungus when cultured at end of experiment. Plants under minus nitrate culture since April 13, 1936. Photographed May 22. Fig. 4, Bonnie Best variety under minus nitrate culture; experiment IV. Plant in righthand pot, manifests chronic type of disease symptoms, is stunted, and has lost all leaves except topmost one. Control plants in pot on left. Plants under minus nitrate culture since April 13, 1936. Plants photographed at same time as those shown in fig. 5.

plants. Symptoms of the diseased plants could be recognized, however, for in the uninoculated plants the tops remained green; whereas, in the diseased ones, yellowing involved all of the leaves, until the



FIGS. 5, 6.—Fig. 5 (left), Bonnie Best, plus nitrate plants, from experiment IV. Righthand plant, center pot, shows acute type of disease picture, with definite wilting; lefthand plant, yellowing and necrosis of leaves approximately half way up the stem. Culture pot on right shows two diseased plants which as yet show no wilting. Control plants in culture pot on left. Plants under nutritional treatment since April 13, 1936. Photographed June 3. Fig. 6 (right), representative control plants of experiment I showing effects of plus and minus nitrate nutrition on vegetative growth of tomato plants. Plus nitrate plants in culture pot on left, minus nitrate (high carbohydrate) plants on right. Photographed July 1, 1935.

plant was finally denuded of all foliage (fig. 4). Stunting of the plants was usually another definite symptom which could be used to differentiate between healthy and diseased plants (figs. 3, 4). In a few cases some of the plants showing the chronic type of symptoms recovered. Both resistant and susceptible minus nitrate plants manifested the same chronic type of disease picture.

In addition to the regular chronic and acute symptoms noted, additional ones were observed. Cessation of growth in the apical meristems (stunting), already mentioned, was a striking feature, and in most cases of regular occurrence (figs. 1-4). In fact growth measurements made at weekly intervals often identified diseased plants before other symptoms were evident. Another striking symptom occurring in many cases as a part of the chronic disease picture, and only in the plus nitrate plants, was that of the intense greening of the topmost leaves. Plants in this stage presented a striking appearance. They were stunted, with the intensely dark green leaves flaccid and slightly drooping but not wilted. This incipient flaccidity of the leaves sometimes (but not usually) developed into a true wilt, death of the leaves coming about through slow necrosis.

In connection with some fumigation in the greenhouses, plants in experiment IV were given two applications of cyanide to control the white fly. After both fumigations it was noticed that the upper leaves were severely burned. The leaves of those plants which showed definite *Fusarium* symptoms (plus nitrate plants), however, were entirely free from cyanide injury. No attempt has been made to explain this observation. Possibly the explanation lies in AH-MET's observations that the rate of transpiration in infected tomato plants was much less than in healthy ones, and that in addition, the diseased plants had their stomata closed.

Plants in experiment IV were checked for the presence or absence of adventitious roots, relative to FISHER's observation that such roots occur on the stems of diseased tomato plants. His observations were found to be diagnostic for plus but not for minus nitrate plants. Adventitious roots have also been noted on diseased petioles of plus nitrate plants.

#### BROWNING OF VASCULAR BUNDLES AS CRITERION OF INFECTION

Various investigators have noted definite browning of the vascular bundles of stems of affected plants and many have used it as a criterion of infection. In the course of the different experiments, observations were made on the presence or absence of vascular browning at the base of the stems. An attempt has been made to

correlate these results with the infection results, which are based on the isolation of the fungus from the stem base.

Results given in table 2 show that out of a total of 275 plants, 144 gave definite browning of the vascular bundles, with 138 (95.8 per cent) positively infected, and 131 gave no evidence of brown vascu-

TABLE 2  
BROWNING AND NON-BROWNING OF VASCULAR BUNDLES  
AS CRITERION OF INFECTION

MATERIAL	CONDITION OF VASCULAR BUNDLES IN RELATION TO INFECTION								
	TOTAL NO. PLANTS	BROWN BUNDLES AT BASE			NO BROWN BUNDLES AT BASE			TOTAL INFECTED	
		No.	NUM- BER IN- FECT- ED*	PER- CENT- AGE IN- FECT- ED*	No.	NUM- BER IN- FECTED	PER- CENT- AGE IN- FECTED	No.	PER- CENTAGE WITH BROWN BUNDLES
Bonnie Best + nitrate.....	69	54	52	96.3	15	5	33.3	57	91.2
Marglobe + ni- trate.....	70	36	33	91.7	34	4	11.8	37	89.0
Total.....	139	90	85	.....	49	9	.....	94	.....
Average.....	.....	.....	.....	94.4	.....	.....	18.3	.....	90.4
Bonnie Best - nitrate.....	66	31	31	100.0	35	17	48.6	48	64.6
Marglobe - ni- trate.....	70	23	22	95.6	47	12	25.5	34	64.7
Total.....	136	54	53	.....	82	29	.....	82	.....
Average.....	.....	.....	.....	98.1	.....	.....	35.4	.....	64.6
Grand total....	275	144	138	.....	131	38	.....	176	.....
Average.....	.....	.....	.....	95.8	.....	.....	29.0	.....	78.4

\* Infected means presence of fungus in tissues of host.

lar bundles. Of these, 38 (29.0 per cent) were infected, however; that is, they yielded fungus upon culturing. One hundred seventy-six infected brown bundle plants and infected non-brown bundle plants were found; 78.4 per cent of these had brown bundles, and 21.6 per cent, no brown bundles. A comparison of the amount of bundle browning and the amount of infection resulting shows no ap-

preciable differences, either for the two varieties or for the different kinds of nutrition. Practically all (95.8 per cent) of the isolations from the base of the stems with brown bundles gave positive infections.

In those plants showing no brown bundles at the base, however, results are different. Minus nitrate plants showed high infection (35.4 per cent), while in the plus nitrate plants only 18.3 per cent of the plants with no browning of vascular bundles were infected.

### Nutritional experiments

#### EXPERIMENT I (TABLE 3)

On March 29, 1935, cuttings of Bonnie Best and Marglobe varieties of tomatoes were transplanted into sterilized sand. The plants were fairly succulent and gave a distinct test for nitrates. Five days after transplanting, all cuttings received plus nitrate solution and were grown continuously under plus nitrate culture for the next 24 days (period ending April 22). At the end of this period approximately half of the plants of each variety were transferred to minus nitrate culture. At the time of changing cultural conditions the average height of all plants was 23.1 cm.

On May 7, all plants were inoculated by pouring fungous inoculum around the roots. At the time of inoculation the plus nitrate plants (especially the tops) were growing vigorously and were fairly succulent. The stems were large and stocky, with an average height of about 53.5 cm. In contrast to the plus nitrate plants, the minus nitrate plants were much less vegetative and succulent, the lower leaves beginning to turn yellow, shrivel, and absciss. The stems were hard, yellowish green in color, and of small diameter. These plants were becoming typical, hard, high carbohydrate plants; they averaged 45.4 cm. in height (fig. 6).

During the elapsed period, from the time of inoculations (May 7) to the end of the experiment (August 1), all plants which exhibited definite *Fusarium* symptoms were removed and used for petri dish isolations. At the end of the experiment (72 days from time of inoculation to removal) all plants were cut off about 5 cm. above the surface of the sand, measured, descriptive notes taken, and they were then brought into the laboratory for isolation studies. In all

TABLE 3

MATERIAL	CONTROLS		INOCULATED PLANTS															
	NUM-BER	AVER-AGE HEIGHT (CM.)	TOTAL NO. PLANTS	WITH EXTERNAL SYMPTOMS*				WITHOUT EXTERNAL SYMPTOMS†				NOT INFECTED‡				TOTAL INFECTED†		
				NUM-BER	PER-CENT-AGE OF TOTAL	AVER-AGE HEIGHT (CM.)	INFECTED‡		NUM-BER	PER-CENT-AGE OF TOTAL	AVER-AGE HEIGHT (CM.)	INFECTED‡						
							NUM-BER	PER-CENT-AGE				NUM-BER	PER-CENT-AGE	NUM-BER	PER-CENT-AGE			
No. I, post-inoculated,    May 7-August 1																		
Bonnie Best + nitrate..	6	182.3	24	4	16.7	132.7	4	100.0	20	83.3	221.0	14	70.0	210.0	6	177.0	18	75.0
Bonnie Best - nitrate..	5	86.0	24	0	.....	.....	0	.....	24	100.0	85.4	15	62.5	84.7	9	86.6	15	62.5
Marglobe + nitrate....	6	181.3	24	0	.....	.....	0	.....	24	100.0	187.0	1	4.2	157.0	23	188.3	1	4.0
Marglobe - nitrate....	5	105.0	24	0	.....	.....	0	.....	24	100.0	91.0	11	45.8	88.5	13	93.2	11	45.8
No. II, post-inoculated,    August 15-October 25																		
Bonnie Best + nitrate..	4	222.0	12	8	66.7	99.4	8	100.0	4	33.3	221.5	3	75.0	213.3	1	246.0	11	91.7
Bonnie Best - nitrate..	2	50.5	9	0	.....	.....	0	.....	9	100.0	52.8	7	77.8	52.4	2	54.0	7	77.8
Marglobe + nitrate....	4	206.0	12	0	.....	.....	0	.....	12	100.0	228.7	8	66.7	224.6	4	234.3	8	66.7
Marglobe - nitrate....	2	40.5	12	0	.....	.....	0	.....	12	100.0	50.7	1	8.3	37.0	11	51.9	1	8.3

\* Characteristic external symptoms of Fusarium injury.

† Apparently healthy plants without externally detectable Fusarium injury, but not without vascular browning.

‡ Infected means presence of fungus in tissues of host.

§ Not infected means no fungus recoverable by culture on agar plate.

|| Substrate inoculated after transplanted plants had rooted.

TABLE 3—Continued

MATERIAL	INOCULATED PLANTS																			
	CONTROLS			WITH EXTERNAL SYMPTOMS*							WITHOUT EXTERNAL SYMPTOMS†							NOT IN- FECTED§		TOTAL IN- FECTION†
	NUM- BER	AVER- AGE HEIGHT (CM.)	TOTAL NO. PLANTS	INFECTED‡			NUM- BER	PER- CENT- AGE OF TOTAL	AVER- AGE HEIGHT (CM.)	PER- CENT- AGE	NUM- BER	PER- CENT- AGE	AVER- AGE HEIGHT (CM.)	NUM- BER	AVER- AGE HEIGHT (CM.)					
				NUM- BER	PER- CENT- AGE	AVER- AGE HEIGHT (CM.)														
																NUM- BER	PER- CENT- AGE	AVER- AGE HEIGHT (CM.)		
No. III, pre-inoculated, ¶ October 18-January 10																				
Bonnie Best + nitrate...	3	131.0	9	5	55.6	19.2	5	100.0	4	44.4	138.0	1	25.0	143.0	3	133.7	6	66.7		
Bonnie Best - nitrate...	2	59.0	9	4	44.5	15.0	0	.....	5	55.5	36.2	0	.....	.....	5	36.2	0	.....		
Marglobe + nitrate....	3	125.0	10	0	.....	.....	0	.....	10	100.0	110.8	7	70.0	118.4	3	123.0	7	70.0		
Marglobe - nitrate....	2	36.0	10	1	10.0	27.0	0	.....	9	90.0	38.1	1	11.1	48.0	8	30.0	1	10.0		
No. IV, pre-inoculated, ¶ April 13-July 2																				
Bonnie Best + nitrate...	6	111.0	24	15	62.5	42.6	14	93.6	9	37.5	93.9	8	88.9	90.1	1	124.0	22	91.7		
Bonnie Best - nitrate...	6	30.9	24	15	62.5	4.5	15	100.0	9	37.5	22.9	9	100.0	22.9	0	.....	24	100.0		
Marglobe + nitrate....	6	100.8	24	7	29.3	41.4	7	100.0	17	70.7	78.3	14	82.5	75.3	3	92.7	21	87.5		
Marglobe - nitrate....	6	24.3	24	10	41.7	2.9	9	90.0	14	58.3	15.7	12	85.7	16.3	2	12.3	21	87.5		

¶ Substrate inoculated prior to transplanting of plants.



cases isolations were made from the bottom of the stem; in some instances from the middle and the top as well. All isolations were incubated at 28° C., and studied microscopically.

#### EXPERIMENT II (TABLE 3)

Starting July 15, 1935, cuttings of Bonnie Best and Marglobe were transplanted into sterilized sand. The cuttings were from plants growing in soil in flats. Owing to the crowded condition in the flats, the plants were spindly (approximately 45 cm. in height), with most of their lower leaves abscised. The upper portions of the plants were vegetative and fairly succulent. For the cuttings, only the upper 25 cm. of the plants was used. About a week after transplanting, the individuals of each variety were divided into approximately two equal lots, one lot of each variety being grown under plus nitrate and the other under minus nitrate culture. The set-up of this experiment varied from the preceding one, in that the experimental plants, which were to be used later for the high carbohydrate, minus nitrate plants, received no nitrates after being transplanted. For the next 30 days (July 15 to August 15) all plants were grown under the cultural conditions already outlined.

Starting August 15, all plants were inoculated as in experiment I (post-inoculated). The plus nitrate plants averaged 48.9 cm. in height, were vegetative, green, and very succulent; the minus nitrate plants averaged 20.1 cm. in height, were spindly, with woody stems and small lower leaves that were turning yellow and beginning to abscise, and with light green and small top leaves. In appearance these plants were typically high carbohydrate (fig. 7).

From the time of inoculation to the end of the experiment (72 days, ending October 25), the plants were carefully watched for symptoms of *Fusarium* damage. At the end of the experiment all plants were removed for isolation studies. In all cases isolations were made from the bottom of the stem, and in this experiment from the middle and top of the plant as well. All fungous growth was checked microscopically.

#### EXPERIMENT III (TABLE 3)

This experiment and the following differ from the preceding in that cuttings were transplanted directly into sand previously inocu-

lated with *F. lycopersici* (pre-inoculation). This procedure shortened the incubation period.

Sand was inoculated on August 23, 1935, and the fungus allowed to incubate until October 18 (55 days). During this period the inoculated sand was watered once daily with tap water, and in addition, each of the inoculated pots received one quart of plus nitrate nutrient solution at weekly intervals.

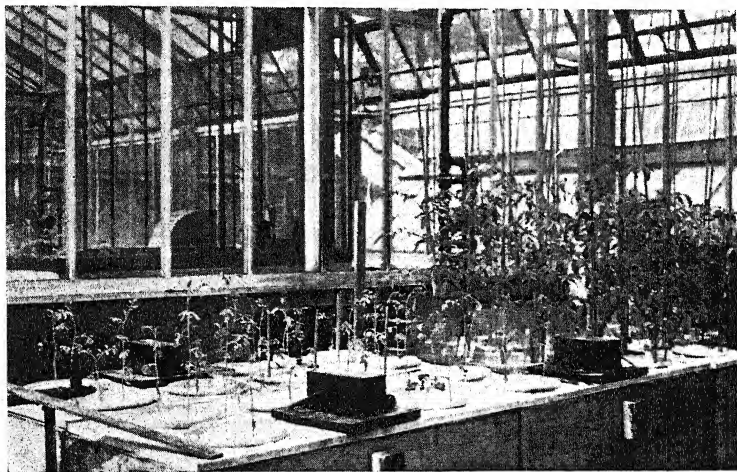


FIG. 7.—Growth of tomato grown in plus and minus nitrate culture (experiment II). Plus nitrate plants on right, minus nitrate plants on left. Plants under nutritional treatment since July 15, 1935. Photographed August 19.

On October 18, cuttings of Bonnie Best and Marglobe were transplanted into the inoculated sand. Plants used as cuttings for the minus nitrate cultures averaged about 45 cm. in height, and were fairly hard near the base. In making these cuttings only the upper 25 cm. of the plant was used. Plants used in the plus nitrate cultures averaged 25–30 cm. in height, were younger than those used in the minus nitrate pots, and more succulent and vegetative. About one week after transplanting, the plants of each variety were divided into approximately two equal lots, one of which was grown under plus nitrate and the other under minus nitrate culture. In this experiment, as in experiment II, plants growing under minus nitrate culture received no nitrates after transplanting; after which the

plants grew well. Those receiving nitrates became typical plus nitrate plants while those receiving no nitrates became typical high carbohydrate plants.

Plants were carefully watched for *Fusarium* symptoms from the time of inoculation (October 18) to end of the experiment (84 days, ending January 10, 1936). As in previous experiments, isolations were made from the bottom of the stems in all cases, and in this experiment mostly from the middle also; a few isolations were made from the top of the stem as well.

#### EXPERIMENT IV (TABLE 3)

As in experiment III, cuttings were transplanted directly into inoculated sand.

The sand was inoculated on April 2, 1936, and the fungus allowed to incubate in it for 12 days. During this period all pots containing the fungus were watered once daily with tap water, and in addition each pot received two quarts of plus nitrate nutrient solution.

On April 13, cuttings of Bonnie Best and Marglobe were transplanted into the inoculated sand. Plants used for cuttings were growing in small pots and averaged 20-25 cm. in height. These plants were highly vegetative and succulent, except near the base. They were blooming, and the bottom leaves were beginning to turn yellow and absciss. They gave a distinct test for nitrates and a rather weak test for starch. Plants were prepared for cuttings in the usual manner. Five days after transplanting, the plants of each variety were divided into two equal lots, one-half of each lot being grown under plus nitrate and the other under minus nitrate culture. Plants grown under minus nitrate culture received no nitrates after transplanting; after which the plants made good growth.

Plants were carefully checked for *Fusarium* symptoms from the time of inoculation to the end of the experiment (79 days, April 13 to July 2). Those plants which showed definite enough symptoms to warrant a tentative diagnosis of the disease were removed during the course of the experiment and used for isolation studies. At the end of the experimental period, all remaining plants were removed and used for isolations from the base of the stem. In addition, isola-

tions were made from the middle and top of the stem as well for most plants.

Results obtained from experiments I to IV show the Bonnie Best (susceptible) plus nitrate plants to be highly infected under both types of inoculation at all seasons of the year. The average infection for all plants in the four experiments amounted to 82.6 per cent; 46.4 per cent displayed symptoms.

Infection in the Marglobe (resistant) variety, under plus nitrate culture, also was high in all except experiment I, in which only 4.0 per cent infection was noted. While an average infection of 52.9 per cent was noted in all experiments, only 4.0 per cent of the plants in experiment I were infected. Only 10.0 per cent of all the inoculated plants showed symptoms, all of which were obtained in experiment IV.

Under minus nitrate culture, the percentage of infection was high for the Bonnie Best variety in all except experiment III, which gave no infection at all. The average infection for all of the inoculated plants was 69.7 per cent. Of the post-inoculated (experiments I and II) plants, 66.7 per cent were infected, while 75.8 per cent of the pre-inoculated (experiments III and IV) were infected. Only 19 plants (28.8 per cent) of all the inoculated plants showed symptoms, 15 of which occurred in experiment IV. In experiment III, four plants out of a total of nine exhibited symptoms, but did not yield the fungus when cultured.

Of inoculated plants of minus nitrate Marglobe variety, 48.6 per cent were infected. In experiment I, with post-inoculated plants, 45.8 per cent were infected. In experiment IV, with pre-inoculated plants, 87.5 per cent were infected. In experiments II and III, an average infection of only 9.1 per cent was obtained. In experiment II the plants were post-inoculated and the period extended from August to October, while in experiment III the plants were pre-inoculated, the period extending from October to January. Production of symptoms was low in these plants; only 11 (15.7 per cent) of the inoculated plants developed symptoms, with all but 1.4 per cent occurring in experiment IV. Two plants, one each from experiments III and IV, exhibited symptoms but were negative when cultured.

An average incubation time of 44 days was noted in the post-in-

oculated plants, while an average incubation time of 25.5 days was noted in the experiments in which the plants were put directly into inoculated sand.

#### SEEDLING TESTS (TABLE 4)

On October 19, 1935, seeds of Bonnie Best and Marglobe were placed in sand which had previously been inoculated, on August 23. From the time of planting to the end of the experiment, lots of each

TABLE 4

INFECTIVITY OF *FUSARIUM LYCOPERSICI* TO BONNIE BEST (SUSCEPTIBLE) AND MARGLOBE (RESISTANT) TOMATO SEEDLINGS GROWN IN INOCULATED SAND UNDER PLUS NITRATE AND MINUS NITRATE CULTURE

MATERIAL	INOCULATED PLANTS														TOTAL INFECTION
	TOTAL NO. PLANTS	WITH SYMPTOMS					WITHOUT SYMPTOMS								
		No.	PER-CENT-AGE	INFECTED*		No.	PER-CENT-AGE	INFECTED*		NOT INFECTED†		No.	PER-CENT-AGE		
				No.	PER-CENT-AGE			No.	PER-CENT-AGE	No.	PER-CENT-AGE				
Bonnie Best + nitrate.....	55	19	34.5	5	26.3	36	65.5	3	8.3	33	91.7	8	14.5		
Bonnie Best - nitrate.....	40	25	62.5	16	64.0	15	37.5	2	13.3	13	86.7	19	47.5		
Marglobe + nitrate.....	63	30	47.6	20	66.7	33	52.4	7	21.2	26	78.8	27	42.8		
Marglobe - nitrate.....	74	21	28.4	13	61.9	53	71.6	1	1.9	52	98.1	14	18.9		

\* Infected means presence of fungus in tissues of host.

† Not infected means no fungus recoverable by culture on agar plates.

variety were grown in plus as well as in minus nitrate culture, the latter receiving no nitrates during the experiment.

Seeds of both varieties germinated well under the two types of nutrition. Following germination, the seedlings made good growth, the plus nitrate ones being the larger. On November 5 the seedlings were thinned to a uniform distribution in the culture pots. Seedlings were watched daily for *Fusarium* symptoms, and plants showing such symptoms were removed and cultured.

Healthy plus nitrate seedlings made the greater growth. The plus nitrate seedlings were taller, averaged 4.1 cm. in height, with about three true leaves. They were very succulent, with weak stems, and had a tendency to fall over. On the other hand the minus nitrate plants were stiffer and stood up much better, averaged 2.6 cm. in height, and usually produced only one true leaf. The experiment was terminated during the period from December 7 to 10, and all remaining seedlings were removed and used for isolations.

Of the inoculated Bonnie Best plus nitrate plants, 14.5 per cent were infected, with 34.5 per cent developing symptoms.

Of the inoculated Marglobe plus nitrate plants, 42.8 per cent were infected, with 47.6 per cent of the seedlings showing symptoms.

In the Bonnie Best minus nitrate inoculated plants, total infection amounted to 47.5 per cent, with 62.5 per cent of the inoculated individuals showing symptoms.

The Marglobe minus nitrate inoculated seedlings gave a total infection of only 18.9 per cent, 28.4 per cent of the inoculated individuals producing symptoms.

### Discussion

It is apparent from the data obtained in these experiments that the so-called susceptibility and resistance of the tomato to *Fusarium* wilt has at least two aspects. One of these is a positive or a negative disposition to infection, that is, to entry of the parasite into the host and its subsequent establishment and spread; the other, a positive or a negative disposition on the part of the tomato to manifest the structural, processal, and behavioral symptoms characteristic of the disease. The former aspect is a measure of the infectivity or non-infectivity of the parasite; the latter of its virulence or avirulence (7).

It is also evident from the results of these experiments that disposition of the tomato to manifest disease symptoms is not unitary. Some infected plants show both vascular discoloration and other symptoms. Others show neither vascular discoloration nor any other disease symptom. Therefore browning of vascular tissues, frequently used as a criterion, is not a measure of the disposition of the tomato to infection.

Because these two aspects of disposition are not clearly differen-

tiated, and because the term infection is indiscriminately used to designate the disease, or some manifestation of symptoms, as well as the state of being contaminated (7), it is difficult to interpret much of the literature dealing with tomato wilt and other wilt diseases.

For the experiments reported here, infection means the state of being contaminated by *Fusarium lycopersici*. It means that the fungus has entered the host, established itself, and spread from the original site of establishment. It does not mean wilt disease in its usual sense. Possibly infection so interpreted involves some injury to cells, so that considered at the cellular level of biological organization, infection of tomato by *F. lycopersici* probably is characterized by microscopically detectable injury and injury reactions; that is, by disease.

The experimental data show that the expression of each of the two aspects of disposition of the tomato is affected by nutrition in both the so-called susceptible and resistant varieties.

CLAYTON (3) states that susceptible varieties of tomatoes grown under minus nitrate conditions "were not infected." If by this he means the fungus was not recoverable in culture, the results of this investigation are at variance with his, because susceptible Bonnie Best plants grown under minus nitrate nutrition yielded fungus upon culturing, and also developed symptoms in one experiment (IV). These results also are at variance with FISHER's (VI), who used browning as a criterion of susceptibility of infection, and reports that the Bonnie Best variety was characterized by lack of infection when grown in absence of nitrates.

FISHER reports that the resistant Marglobe showed practically no infection when grown in absence of nitrates. The results of this study are at variance with his, in that in two experiments (I and IV) Marglobe showed infection when grown without nitrates. FISHER reports that Marglobe developed infection when grown with an excess of nitrates. In the experiment reported here, frequency of infection was high in both the resistant and susceptible varieties (except in experiment I) when grown in plus nitrate nutrition. In experiment I Marglobe was resistant to infection.

The writer is unable to advance any plausible explanation for the exceptions noted in experiments I-IV. Average frequency of infec-

tion of Marglobe plants was higher when the plants were transplanted into pre-inoculated sand (experiments III and IV) than when inoculation was effected by working the fungus into the sand around established plants (experiments I and II). Possibly severe trauma and other injuries incident to cutting and transplanting disposed the plants to infection. Bonnie Best also showed a higher average frequency of infection in the experiments (III and IV) involving cutting and transplanting into previously inoculated sand. It is possible that the amount of fungus present was greater and more effective in the pre-inoculated sand experiments. Possibly the age of the plants also contributed to injury. These transplanted plants were younger and more succulent at the base than the established plants. Possibly differences in air temperature, humidity, and sunshine were factors, through different influences on the nutritional state of the experimental plants at different seasons of the year. Generally speaking, both plus and minus nitrate plants contain more carbohydrates (starch) during the late spring, summer, and early fall months than during the winter months. Plus nitrate plants are high in nitrates at all seasons of the year, while minus nitrate plants contain no readily demonstrable nitrates at any season of the year.

I am also not prepared to explain the differences in frequency of expression of symptoms noted in experiments I-IV. Only Bonnie Best plants when post-inoculated were disposed to symptom manifestation in plus nitrate culture. In pre-inoculation experiments, however, both Bonnie Best (in experiments III and IV) and Marglobe (in experiment IV) were disposed to symptom manifestation in plus nitrate culture. The former alone manifested true wilting in addition to the chronic type of symptoms; the latter manifested only the chronic type. The same factors that were responsible for irregularities in the infection data may have been responsible for deviations in the manifestation of symptoms. Pre-inoculations and post-inoculations also affected the incubation time, symptoms appearing in 25 days for the first and in 44 days for the latter.

These experiments reveal discrepancies between frequency of browning in the stem or manifestation of other symptoms, and between frequency of recovery of the fungus by culture. Plants with brown bundles (experiments III and IV) did not invariably yield the



fungus in culture. This does not mean that they were not infected, because presence or absence of the fungus in the root system was not determined by culture. It is well known that browning may occur in advance of the site of the fungus. A decisive answer to this problem would entail culture of the entire root system of every plant.

The other discrepancy, namely the recovery of fungus from plants showing no symptoms whatsoever in the aerial parts, indicates manifestation of low virulence on the part of the parasite and of high resistance to virulence on the part of the plant. This discrepancy was most marked in plants under minus nitrate nutrition.

Possibly the discrepancy between infection as determined by culture on the one hand and by browning on the other would have been less if microscopically detectable browning had been used as a criterion of presence of the fungus. FISHER used the microscopic criterion.

### Summary

1. Experiments were conducted to study the relation of nutrition of the tomato as a factor in its disposition to the infectivity and virulence of *Fusarium lycopersici*. Bonnie Best (susceptible) and Marglobe (resistant) varieties were tested. Nutrition was varied by the application or non-application of nitrates under otherwise identical conditions. The substrate was inoculated both previous (pre-inoculation) to and subsequent (post-inoculation) to the establishment of the plants.

2. Recovery of the fungus from the base of the stem was used as a criterion of infectivity and infection. Manifestation of macroscopically detectable symptoms was used as a criterion of virulence and of pathic effects in the host.

3. Results of the nutritional experiments revealed (a) a high frequency of infection of both resistant and susceptible tomato plants under minus nitrate nutrition; (b) a low frequency of symptoms in plants under minus nitrate nutrition; (c) no wilting in the so-called resistant Marglobe under either type of nutrition; (d) seedlings of the resistant variety under either plus or minus nitrate culture read-

ily infected and producing symptoms identical with those of the susceptible variety.

4. Type of inoculation and age of the plant are important factors in infection and symptom production: (a) Pre-inoculation of sand led to a higher frequency of infection and symptom production. (b) Post-inoculation of sand under minus nitrate culture did not lead to the development of any plant symptoms, even in infected plants. (c) Bonnie Best variety under plus nitrate nutrition showed a high frequency of infection and of symptom production of both the chronic and the acute type, at all seasons of the year, when either pre-inoculation or post-inoculation was used. (d) Pre-inoculation practically halved the incubation period.

5. Browning of vascular bundles of the stem alone is an inadequate criterion of infection. Even under minus nitrate nutrition, 35.4 per cent of the infected plants did not manifest brown bundles at the base of the stem.

The writer gratefully acknowledges the valuable assistance and criticisms of members of the staff of the Botany Department at the University of Chicago during the progress of this work.

TALLULAH, LOUISIANA

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# EQUIPMENT FOR GROWING PLANTS IN NITROGEN FIXATION STUDIES

C. A. LUDWIG

(WITH THREE FIGURES)

## Introduction

In certain nitrogen fixation and related studies in this laboratory during the last five or six years (1, 14, 15, 16), the writer has been using two kinds of apparatus for growing the plants in artificial light. The first type of apparatus produces good growth of plants in considerable numbers under conditions which are rather closely reproducible. However, many of the refinements of control necessary when study of the light relations of plants is undertaken have been omitted, since the purpose was merely to grow plants in fairly large numbers with some approach to "normal" characteristics. The apparatus is easily constructed, inexpensive, and requires little attention in operation. A second type of apparatus has been used when it was desirable to grow plants under more rigidly controlled biological or atmospheric conditions. The following description of the equipment concerned has been prepared in response to the suggestion that it would prove helpful to others with a similar problem.

## Apparatus no. 1

The apparatus, the essential structural details and the general appearance of which are shown in figures 1 and 2, differs from previously described installations chiefly in that all types of light filters, air conditioning devices, etc., for improving the spectral balance of the light and for preventing high air temperature and aridity have been discarded. Instead some mercury vapor lamps have been added to improve the spectral balance and a water bath has been introduced to keep root temperatures from becoming too high. The apparatus consists of a table supporting the water bath, in which the culture jars are placed, an overhead bank of lights, and the necessary accessory equipment for operation.

The water bath is a copper lined wooden tank. The water enters from the city water system through small holes drilled in a pipe which lies on the bottom of the tank and against the wall on three sides. It leaves through an overflow pipe which can be adjusted as to height so as to give within limits any desired depth of water. The temperature and rate of admission of the cold water determine the

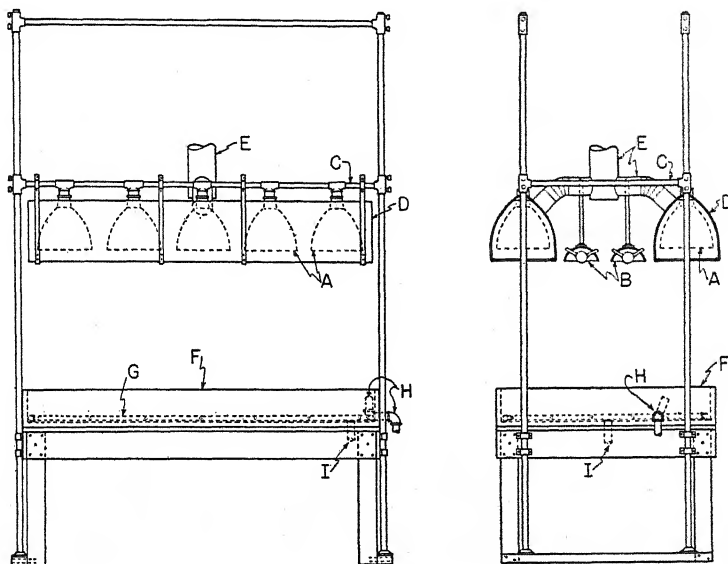
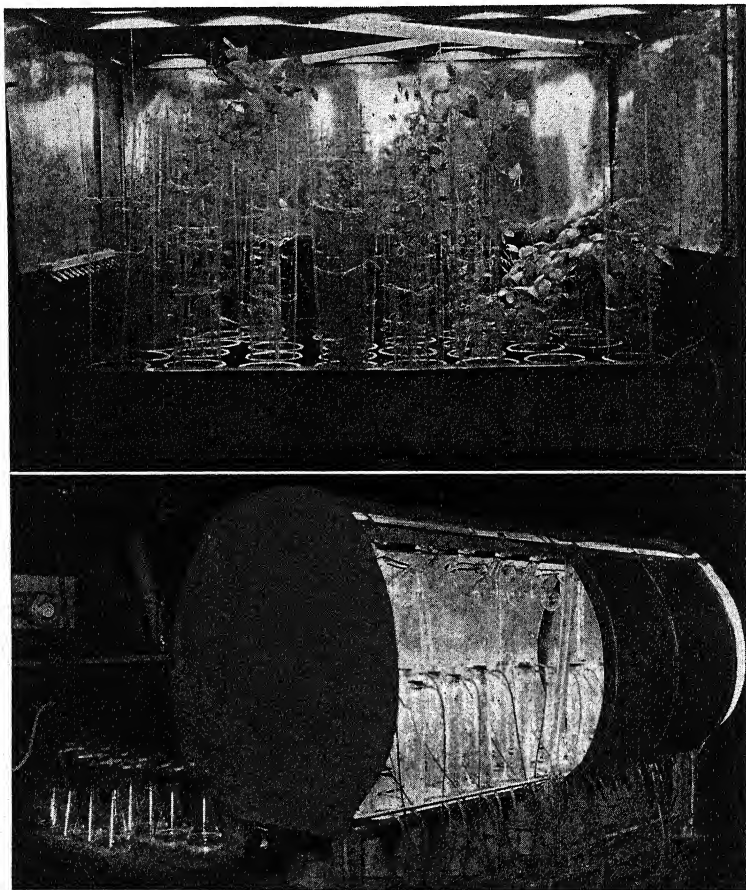


FIG. 1.—Drawing of side and end of plant culture apparatus: *A*, enameled reflectors; *B*, mercury vapor lamps; *C*, adjustable framework supporting the lamps; *D*, galvanized iron hood surrounding reflectors; *E*, air removal pipes connected with hoods and suction fan; *F*, water bath; *G*, perforated water inlet pipe; *H*, constant level device; *I*, drain. Water pump and tin plate reflectors on sides and ends not shown.

temperature of the bath. As the rate of admission is constant for considerable periods of time, the temperature goes down at night and rises during the day, and thermostatic control of temperature would be preferable for many kinds of investigation. In the summer the incoming jets of water are strong enough to cause mixing and prevent thermal stratification in the tank. In the winter, owing to the low temperature of the tap water, the amount allowed to enter is too small to produce jets; and mixing is produced by a circulating pump

(not shown in the figures) from which the water is reintroduced as two jets near opposite corners of the tank.

The light is produced by ten 500 watt gas filled tungsten filament



FIGS. 2, 3.—Fig. 2 (above), side view of plant culture apparatus, with the nearer tin plate reflector removed to show growing plants in place; fig. 3 (below), apparatus for growing a number of plant cultures at one time by artificial light under biologically controlled conditions.

lamps and two 450 watt mercury vapor lamps arranged as shown in figure 1. They are adjustable in height to approximately 1.1 m. (43 in.) above the surface of the water; a greater height would be an

advantage at times. The incandescent lamps can be tilted through a limited angle for equalizing the light over the table. All lamps have white enameled reflectors; additional vertical tin plate reflectors (not shown in figure 1) at the ends and sides of the apparatus extend downward to within about 27 cm. (10.5 in.) of the top of the water tank. The side reflectors are removable for allowing access to the cultures. A time clock, operating through a suitable relay, turns the lights on and off. Any desired relative length of "day" and "night" with a total 24-hour period can be secured.

The light intensity attained a few inches above the surface of the water is about 2200 foot candles<sup>1</sup> (23,700 lux), but varying with the age of the light bulbs in use, the voltage in the line, and the height of the lamps above the water. If this be compared with daylight in the open it may at first seem rather low, since in the latitude of Washington this latter value at noon occasionally exceeds 10,000 foot candles (107,600 lux), and has been known to reach 14,000 foot candles (150,600 lux). The mean intensity, however, during the daily period of illumination for June at Washington, D.C., as an average of 18 years is only approximately 3600 foot candles (39,000 lux). The corresponding figure for December is 2100 foot candles<sup>2</sup> (22,600 lux). It is thus seen that the intensity secured in the apparatus is of a magnitude comparable with out-of-door intensities in which good growth occurs when other factors are favorable. Experience indicates, however, that a greater intensity would often be more favorable, and that certainly it cannot be reduced greatly, for instance by as much as half, without harmful results on the plants.

The spectral quality of the light is of course different from that of sunlight, since so much more of the emission from filament lamps is in the infra-red than is the case with sunlight and since the emission of mercury vapor lamps is in narrow bands. In view of the fact that JOHNSTON (13) has found an excess of infra-red radiation to be injurious to some plants under certain conditions, it might be expected that injuries would occur in this case. No serious bad effects

<sup>1</sup> Intensity measured by Weston photronic cell and by Sharpe-Millar photometer.

<sup>2</sup> Information from the records of the U.S. Weather Bureau. Furnished by Mr. I. F. HAND.

have been noticed which seem to be attributable to this source, however, although the internodes are often longer than normal, especially while the plants are still very young. Possibly the enrichment of the blue and near ultraviolet has prevented real injury in this case. The importance of the shorter wave length portion of the light is further suggested by the results of DASTUR and MEHTA (7), who have reported that a proper balance of the spectral elements is necessary for maximum photosynthesis.

The water bath prevents excessive temperatures from developing around the plant roots. The dissipation of the considerable amount of heat produced by incandescent lamps has been one of the greatest problems connected with their use for growing plants (2, 5, 8, 17, 18, 19); in at least one recent case it has been found sufficient for heating a well insulated small greenhouse (3). One of the points carefully considered in the original design of the apparatus was therefore that of adequate removal of excess heat, in spite of the fact that some workers had already shown that plants can sometimes be grown in artificial light without special measures for heat dissipation (9, 10, 11). A heat dissipation device was tried which was composed of a heat absorbing glass filter, cooled by a current of air drawn over it by a suction fan. This filter reduced the intensity of the light too much for economical operation and did not remove the heat as efficiently as was hoped. Experiments soon showed that most ordinary plants can stand without damage the highest air temperatures which are reached in the apparatus without any light filters at all, provided the roots are kept cool. The adoption of the water bath and rejection of the filters accordingly followed. Air temperatures now usually approximate 30° C., and except near the lamps have not been observed above 37°, which is often surpassed in the open. The plants seldom show heat injury until they grow to within about 6 inches (15 cm.) or less from the naked bulb. The air drainage system has been retained as a useful but unessential aid in temperature control. Recently it became desirable to use a much higher light intensity for one experiment. This was attained by using 1000 watt instead of 500 watt lamps and lowering them closer to the plants. Under these conditions the plants showed heat injury. The tin plate sides were then removed and a current of air from a fan was

blown across the table during the remainder of the experiment. No further heat injury occurred.

The importance of keeping the plant roots cool should be emphasized. When the apparatus was first constructed and before the water bath was added, many plants, especially those which can be classed as cool weather plants, did not thrive. A trial with part of the jars standing in a shallow tray of water showed a considerable improvement. The tank was then installed, in which the water is maintained at a depth as great as that of the sand in the jars, or slightly greater, and the improvement was still greater. The tap water in Washington sometimes reaches a temperature as low as 5° C. in the winter, so that it is then possible to keep the roots as cool as any ordinary plant requires. In the summer the tap water exceeds 25° C. much of the time, however, so that it is then impossible to cool the sand below this figure. As best results seem to be secured by a temperature of 20° to 25°, a cooling device for the water in the summer would be an advantage.

During the earlier part of the work attention was concentrated on the growing of subtropical plants, since it was supposed that the high air temperatures would cause plants typical of higher latitudes to fail. Recent experience has shown, however, that alfalfa, sweet clover, sweet peas, garden peas, and other such plants will do well at the temperatures concerned. In fact, in one experiment alfalfa, sweet clover, and sweet peas grew better in the apparatus than they did in sunlight, where, however, the sand became very warm. Cowpeas, on the contrary, did better in the sunlight. It is now believed that most of the ordinary spring or fall (cool weather) crops can be grown satisfactorily. From some standpoints they may be even better suited to the conditions produced in the apparatus than are plants of lower latitudes.

Alfalfa, soybean, garden pea, garden bean, ragweed (*Ambrosia artemisiifolia* L.), pigweed (*Amaranthus retroflexus* L.), partridge pea (*Cassia chamaecrista* L.), and other plants blossomed and produced seed. Sweet pea, sweet clover (*Melilotus alba* Desr.), white clover, alsike clover, red clover, pokeberry (*Phytolacca decandra* L.), golden rod (*Solidago* sp.), millet, Sudan grass, and other plants blossomed but either did not mature seed or data concerning the point were not



secured. Cowpea, lespedeza (*Lespedeza sericea* L. and *L. stipulacea* Maxim.), *Crotalaria spectabilis*, vetch (*Vicia villosa* Roth), and timothy grew well but under the conditions involved did not bloom. An idea of the growth of the plants can be obtained from figure 2, where can be seen alfalfa, sweet pea, soybean, garden bean, cowpea, and Korean lespedeza plants.

The behavior of the plants was very nearly normal except, as already mentioned, for a somewhat greater internode length, especially in the seedling stage, and, as in cowpea, for a slightly variant morphology in other respects. HIRTH (12) has reported a similar lengthening of the internodes but he appears to have worked with a lower light intensity. In the present work the addition of the mercury arc radiation was found to improve the plants in this respect. It is therefore likely that the undue lengthening still noted is due at least in part to a lack of balance between long wave and short wave radiation. Further improvement in plant growth could doubtless be obtained by increasing the total light intensity or, likely, by increasing the blueviolet-ultraviolet portion.

There are no actual figures on the operating costs of the apparatus except such as can be figured from the capacity of the lamps. Ten 500 watt lamps and two 450 watt lamps will use approximately 5.9 kw. per hour. At 2.5 cents per kw. hour, which is the approximate price paid for electric current at this laboratory, the cost is 14.75 cents per hour or \$1.76 for a 12-hour day. A total of \$2.00 per day as an estimate to cover all operating costs except repairs and replacement of lamps would probably be more than ample at the given kw. hour rate. If it should become practical to substitute glow lamps for the incandescent ones in installations of this kind, as may happen before many years (4), the change would reduce the cost of current very materially, as the efficiency of this type of lamp is much greater than that of incandescent lamps.

#### Apparatus no. 2

This apparatus is an adaptation of a growth chamber first used at the Smithsonian Institution (6, p. 128, and pl. 1, fig. 1) in studies of photosynthesis by wheat. The unit for a single culture as used in this laboratory consists of a double walled, cylindrical culture cham-

ber of glass mounted in a wide-mouthed Erlenmeyer flask by means of a rubber stopper with a large hole in it. A tube to the inner chamber at the base serves to introduce air, which may be modified in any way desired. A glass tube through a hole in a rubber stopper at the top permits the air to escape. The space between the inner and outer walls contains flowing water, which serves to keep the interior cool and to filter out much of the infra-red portion of the radiation. Only small leaved plants can be grown with this equipment, as there are mechanical limits to the size of apparatus that can be made at reasonable cost.

In practice, the apparatus, with the flask containing the moist sand and its culture salts, is sterilized in the autoclave. The seeds are sterilized and germinated outside; they are then planted with aseptic procedure, covered with a little sterile sand, and aerated with sterile, moist air throughout the growth period. It is not necessary to add water at any time, as the chamber acts like a reflux condenser. For ordinary plant culture it is sufficient to circulate tap water; for temperature studies it would of course be necessary to have thermostatic control.

It has been found convenient to surround these cylindrical chambers with a housing of tin plate in order to conserve the light, leaving the flasks with the sand below the housing. Figure 3 shows such a housing in use. It would improve the design to put the lights lower so as to increase the light intensity available from a given consumption of electricity.

In this chamber vetch and wheat grow excellently. The only other plant tried, one of the lespedezas, did not do so well. The cause of its failure may have been unsuitable culture salts or wrong temperature. There is thus far no reason to think that the cause was in the apparatus itself.

### Summary

1. Two types of equipment used in symbiotic nitrogen fixation and related studies are described. One of these consists of a shallow water bath, an overhead bank of lights, and the necessary accessory equipment for operation. The main features in which it differs from installations previously described are in discarding light filters and air conditioning devices for control of spectral balance and air tem-

peratures, and in substituting mercury vapor lamps and a water bath. It was found that most plants will grow well at the highest air temperatures reached if the spectral balance is fairly good and the roots do not become too warm. The intensity of illumination secured is usually about 1500 to 2200 foot candles (16,100 to 21,500 lux). With 1000 watt lamps over 4000 foot candles (43,000 lux) has been attained. Most plants grow in a practically normal manner in the apparatus.

2. The second apparatus consists of a unit composed of a water jacketed glass culture chamber mounted by means of a rubber stopper in a wide mouthed Erlenmeyer flask which contains the material in which the roots grow. A number of such units are assembled in a tin plate housing to conserve the light. Good growth of small leaved plants under biologically controlled conditions is easy to secure with the apparatus.

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# EXPERIMENTS CONCERNING DIFFUSION OF NITROGENOUS COMPOUNDS FROM HEALTHY LEGUME NODULES OR ROOTS

C. A. LUDWIG AND FRANKLIN E. ALLISON

(WITH TWO FIGURES)

## Introduction

It has long been common knowledge that the cultivation of legumes followed by their addition to the soil increases soil nitrogen. Occasionally also it has been claimed that the soil can be enriched in nitrogen before the death of the legumes and even while they are young and growing vigorously. Most of the recorded experiments on this point have been inconclusive, although sometimes very favorable to the idea. Recently, however, VIRTANEN and his associates (11, 12, 13, 14, 16, 17, 18, 19, 20, 21, 22), of Helsingfors, Finland, have carried out a series of rigidly controlled experiments in which they found, not only that nitrogenous compounds are given off into nitrogen-free sand in this way but that enough of such compounds are given off to permit normal development of non-legumes growing in the same sand. This whole subject has recently been reviewed in detail by NICOL (7). The present work is along the same line and represents chiefly an attempt to duplicate their results, mostly with other crop plants and under somewhat different conditions. A preliminary statement was presented at the New York meeting of the Society of American Bacteriologists in December, 1935, and an abstract has been published in connection with that meeting. (6).

## Methods

Most of the cultures have been grown with artificial light in an apparatus described in this issue (5) which has been found suitable for the growth of many plants. In this apparatus the source of the light is ten 500 watt Mazda incandescent lamps and two 450 watt mercury vapor lamps in glass, which together give an intensity of about 2200 foot candles (23,700 lux) at the surface of the sand in

which the plants grow. Some other cultures were grown in a modified coldframe in the open. Culture vessels have been for the most part approximately 4-liter glazed earthenware jars containing 4.5 kilos of an almost nitrogen free washed quartz sand to which minerals except nitrogen were added, either in solution or as a coarse powder.

Several mineral mixtures were tried but most of the work was done with one of the following three:

1. A fertilizer mixture used by GIÖBEL (2) for growing alfalfa, and modifications of the same. This proved unsuitable, after thorough trial, for the growth of most plants, and has been discarded except for a special case mentioned later.

2. A solution used by VIRTANEN and associates (17, p. 63) for growing legumes. This contains:

MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	1.2 gm.	KH <sub>2</sub> PO <sub>4</sub> .....	1.4 gm.
KCl.....	1.5	Water.....	20 liters

As used by VIRTANEN it contains a trace of ferric chloride also, but as used here this was omitted and instead 0.6 mg. per culture of iron combined with humic acid extracted from soil was supplied at planting time. One third gram of tricalcium phosphate per kilogram of sand is mixed dry with the sand previous to planting and the solution is used to water the plants.

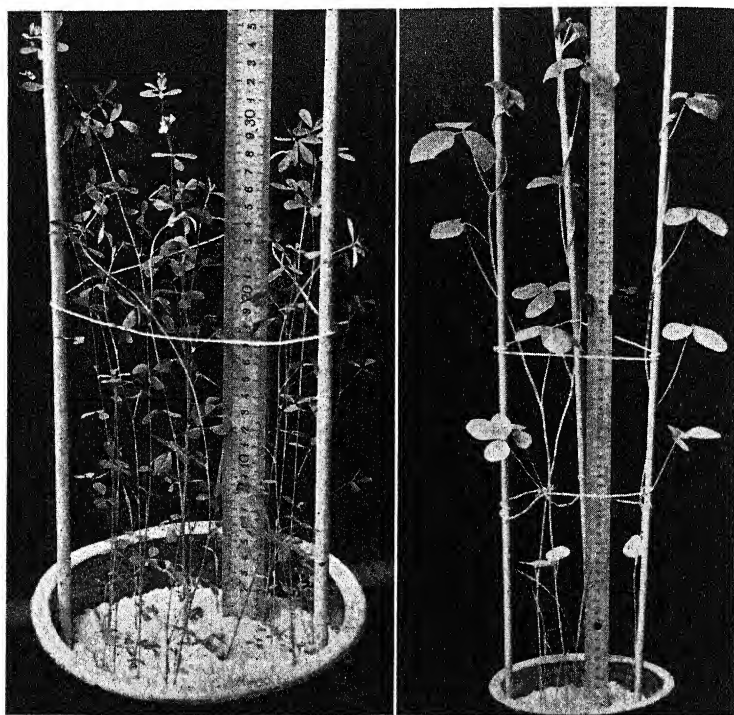
3. A solution used by THORNTON (8) and associates, also for growing legumes. As used here it contains:

KCl.....	0.74 gm.	NaCl.....	0.5 gm.
K <sub>2</sub> HPO <sub>4</sub> .....	0.3	CaSO <sub>4</sub> .....	0.5
KH <sub>2</sub> PO <sub>4</sub> .....	0.3	Water.....	1 liter
MgSO <sub>4</sub> ·7H <sub>2</sub> O...	0.5		

Iron was added at the rate of 0.6 mg. per culture in combination with humic acid extracted from soil. The solution is used to moisten the sand at the beginning of an experiment and more is added later if the growth of the plants seems to make it necessary. This solution proved to be the most successful of any tried thus far.

In most cases 1 mg. per culture of boron as sodium borate was also added. It was sometimes necessary to repeat the addition of boron during the course of the growth period in order to prevent death of the growing buds.

Cultures grown in this way are not sterile, although in some cases an initial sterilization of sand and seeds was made. Sterilization is probably of small importance, since VIRTANEN obtained his results with both sterile and non-sterile cultures. Common contaminants



FIGS. 1, 2.—Fig. 1 (left), alfalfa, Hungarian millet, and timothy culture 35 days after planting the alfalfa. Fig. 2 (right), soybean, Sudan grass, and Hungarian millet culture 33 days after planting the soybeans. In both cases the grasses were planted after the legumes were well started. Note bloom on one alfalfa plant and small size of the grasses in each culture.

in the present cultures were one or more species of algae, some of which appear to have been nitrogen fixing forms; but the results indicate that the amount of nitrogen fixed was never enough to cast doubt on the conclusions. However, some cultures which were sterile as to organisms other than the nodule bacteria have been carried out. These were conducted in double walled, water cooled growth cham-

bers similar to that used at the Smithsonian Institution (1, 3, 5) for studies in photosynthesis. Only small leaved plants can be grown in these chambers; vetch and wheat were used in this work. The chambers were mounted in wide mouthed Erlenmeyer flasks each containing about 4 kg. of sand. The sand contained 1.25 gm. per kilogram of a modified GRÖBEL's fertilizer mixture. This contains the following substances, finely ground together:

Ground marble.....	4 parts	KCl.....	5 parts
Superphosphate.....	20	MgSO <sub>4</sub> .7H <sub>2</sub> O.....	2

Iron was added separately as iron humate at the rate of 0.3 mg. Fe per culture. The apparatus was sterilized for one to two hours in the autoclave at 18-19 pounds' pressure. The air which was passed through the chambers was enriched to about 0.5 per cent with CO<sub>2</sub> to improve photosynthesis, and was bubbled through strong sulphuric acid and a mercuric chloride solution to remove any combined nitrogen, to sterilize it, and to humidify it. Seeds were sterilized by treating for 5 minutes or more with concentrated H<sub>2</sub>SO<sub>4</sub>, rinsing a few times in tap water, immersing in 1/1000 HgCl<sub>2</sub> solution for 5 minutes or a little more, and rinsing six times or more with sterile water.

The moisture content usually desired for the cultures was about 12 per cent. If more water is added it flows to the bottom of the jar or flask and does not increase appreciably the moisture content of the bulk of the sand. It should be noted, however, that much of the time the moisture content varies greatly in the cultures open to the air, since as soon as the plants attain a considerable size they transpire water so rapidly that a jar often loses in one day almost or quite as much water as is necessary to make a moisture content of 12 per cent. It was not often practical to water more than once a day, and of course after plants reached this stage it was very impractical to attempt to regulate moisture closely by bringing the jars to weight occasionally, although this was done while the plants were small. With the sterile cultures it was not necessary to add water at any time, as the growth chamber acted as a reflux condenser and returned the transpired water to the sand.

The following legume plants have been used at one time or an-



other: cowpea (*Vigna sinensis* (L.) Endl.), soybean (*Soja max* (L.) Piper), alfalfa (*Medicago sativa* L.), vetch (*Vicia villosa* Roth), sweet pea (*Lathyrus odoratus* L.), sweet clover (*Melilotus alba* Desr.), red clover (*Trifolium pratense* L.), pea (*Pisum sativum* L.), bean (*Phaseolus vulgaris* L.), and Korean lespedeza (*Lespedeza stipulacea* Maxim.). The following non-legumes have been used: Sudan grass (*Sorghum vulgare sudanense* (Piper) Hitchc.), millet (species uncertain), Hungarian millet (*Setaria italica* (L.) Beaüv.), pigweed (*Amaranthus retroflexus* L.), wheat (*Triticum aestivum* L.), timothy (*Phleum pratense* L.), oats (*Avena sativa* L.), barley (*Hordeum vulgare* L.), and Italian rye grass (*Lolium multiflorum* Lam.).

At the close of the growth period the plants were harvested and separated into legume tops, non-legume tops, and (where possible) legume roots and non-legume roots. In many cases, however, it was impractical to separate the roots of the two kinds of plants and they were analyzed together. The airdry weight of the tops was usually taken but that of the roots was omitted on account of the clinging sand. These weights are omitted in the following discussion, however, as they parallel fairly closely the nitrogen found. Sand extracts were made in the earlier experiments by putting the sand in a glass cylinder and allowing cold water to percolate through. In the later extractions (experiments VI, VII, and VIII) a special apparatus was used in which an initial cold water extraction was followed by intermittent extraction for several hours with water at nearly the boiling point. Nitrogen determinations were by the Kjeldahl method.

### Results

The results, except as regards total nitrogen in the sand, are given in table 1. It will be observed that, with one exception, whether the criterion adopted be the nitrogen accumulated by the non-legumes or the water extractable nitrogen in the sand, the indications are the same throughout the entire series of experiments; namely, that under the conditions concerned little or no nitrogenous material was excreted from legume nodules or roots into the substratum. In fact, the non-legumes grown in association with legumes were usually smaller and accumulated less nitrogen than the very small ones grown alone. The amount of water extractable nitrogen in the sand

TABLE 1

NITROGEN ACCUMULATION BY NON-LEGUMES GROWING IN ASSOCIATION WITH LEGUMES IN DIFFERENT EXTERNAL CONDITIONS

Exp. No.	No. of cultures	Species and approximate no. of plants used		Mineral mixture or solution used	Approximate duration of experiment (months)	Nitrogen added in seeds		N <sub>2</sub> fixed <sup>a</sup> (mg.)	N in non-legumes less than in seeds planted			Remarks
		Legume	Non-legume			Legume (mg.)	Non-legume (mg.)		Control (mg.)	Experimental <sup>a</sup> (mg.)	Gain or loss by association <sup>a</sup> (mg.)	
II	2	1 cowpea	pigeeweed, Sudan <sup>e</sup> grass, millet	Giöbel <sup>d</sup>	8	5.7	1.0	335-433	.....	.....	.....	.....
	1	2 cowpea	1 pigweed, 1 Sudan grass, 1 millet			11.06	0.3	43	0.54 <sup>f</sup>	0.05 <sup>f</sup>	-0.49	.....
III	1	2 cowpea	" "	Giöbel <sup>d</sup>	3.7	11.86	0.3	51	1.13 <sup>f</sup>	0.07 <sup>f</sup>	-1.06	.....
	1	2 cowpea, uninoculated	" "	Giöbel <sup>d</sup>	3.5	11.86	0.3	3	1.13 <sup>f</sup>	0.54 <sup>f</sup>	-0.59	.....
	1	2 cowpea	" "	Hopkins <sup>d</sup>	3.3	10.30	0.3	30-66	0.21 <sup>f</sup>	0.03 <sup>f</sup>	-0.18 to -0.05	.....
	1	2 cowpea, uninoculated	" "	"	3.3	10.30	0.3	3	0.21 <sup>f</sup>	0.08 <sup>f</sup>	-0.13	.....
IV	2	cowpea	2 pigweed, 1 Sudan grass, 1 millet	Modified <sup>d</sup> Giöbel	5.5	.....	.....	82-96	2.8	8	.....	.....

<sup>a</sup> Where more than one value is concerned the two extremes are given rather than the mean.<sup>b</sup> One culture grown in natural light for a time.<sup>c</sup> Planted at beginning of seventh month, after fixation was well established.<sup>d</sup> Giöbel's fertilizer mixture, as used in experiments II and III, gave a slightly alkaline reaction, usually pH 7.0 to 8.0 but more often nearer 8.0 than 7.0. As modified and used in experiments IV, V, VI, and VII its reaction was about neutral. The Hopkins solution gave a reaction usually about pH 7.3 to 7.8; Thornton solution in sand gave a reaction of about neutrality.<sup>e</sup> Tops only.<sup>f</sup> Tops only, not corrected for nitrogen added in seed.<sup>g</sup> Through an error, the nitrogen in the legume and in the non-legume plants was not determined separately. However, the mean air-dry weight of the tops of the non-legumes grown in association with cowpeas was 0.111 gm. while corresponding weight of plants grown alone was 0.302 gm.; evidently no significant absorption of nitrogen from the legumes can have taken place.

TABLE 1—Continued

Exp. No.	No. of Cul- tures	Species and Approximate No. of Plants Used		Mineral Mix- ture or Solution Used	Approximate Duration of Ex- periment (Months)	Nitrogen Added in Seeds		N in Non-Legumes Less That in Seeds Planted		Water <sup>a</sup> Soluble N in Sand at Close (mg.)	Remarks
		Legume	Non-Legume			Leg- umes (mg.)	Non- leg- ume (mg.)	Con- trol (mg.)	Experi- mental <sup>a</sup> (mg.)	Gain or Loss by Association <sup>a</sup> (mg.)	
Vh	2	2 vetch	2 wheat	Gibbel <sup>d</sup>	2.3-3.5	2.88	1.8	67-103	1.3-11.1	9.5-10.4	.....
VIb <sup>1</sup>	0	2 vetch	2 wheat	Gibbel <sup>d</sup>	3-3.5	1.41	1.88	46-114	0.7	1.8	.....
	1	2 vetch, un- inoculated	2 wheat	Gibbel <sup>d</sup>	.....	1.41	1.88	11	0.7	0.4	1.1 -0.2
VIIb	2	2 vetch	2 wheat	Gibbel <sup>d</sup>	2.5	1.30	1.88	133	2.0-2.3	6.0-6.1	.....
VIII	8	9 alfalfa	4 Sudan grass	Thornton <sup>d</sup>	4.5-5	.....	.....	55-301	1.2-3.3 <sup>f</sup>	9.9-17.1 <sup>m</sup>	.....
IX	5	15 alfalfa	8 timothy, 4 Hungarian millet, and 4 Sudan grass	Thornton <sup>d</sup> and Virtanen	4	3.09	1.10	160-380	3.5	0.0-2.1	.....
	5	15 alfalfa	8 timothy and 4 Hungarian millet	"	4	3.09	1.10	280-387	3.5	0.0-0.4	Aerated
	4 <sup>n</sup>	15 alfalfa	8 timothy, 4 Hungarian millet, and 4 Sudan grass	"	3.7	3.09	0.31	43-08	5.0	0.8-3.0	Sunlight
	2	15 sweet clover	4 Sudan grass	"	4	2.83	1.10	173-204	3.5	1.0-1.8	.....
	2 <sup>m</sup>	15 sweet clover	8 timothy and 4 Hungarian millet	"	2.2	2.83	0.31	70 <sup>o</sup>	5.0	0.0-1.5	Sunlight
	5 <sup>n</sup>	6 vetch	5 Hungarian millet and 1 oat	"	2	14.80	2.08	12-75	6.0	0.6-1.1	Sunlight
	0	4 sweet pea	2 Hungarian millet and 2 Sudan grass	"	3.3	21.02	0.99	83-125	4.8	2.5-4.9	.....
	5 <sup>n</sup>	4 sweet pea	"	"	3.3	21.02	0.99	38-90	4.8	1.7-5.6	Aerated
	5 <sup>n</sup>	4 sweet pea	"	"	2	21.02	0.99	8-16	3.2	0.7-4.2	Sunlight
	2 <sup>n</sup>	3 cowpea	2 Hungarian millet and 4 Sudan grass	"	3.7	24.71	1.39	430-514	4.8	0.1-0.2	.....
						24.71	0.60	901-906	3.2	0.2-1.0	Sunlight

<sup>b</sup> Cultures sterile except for presence of nodule bacteria.<sup>i</sup> Light intensity for these cultures only a little more than half as great as for nos. V and VII.<sup>j</sup> Apparent error in subtraction is due to carrying out the calculations to further digits and rounding off the final results.<sup>k</sup> Extraction started with cold and finished with hot water. The following additional values can be given: one culture, inoculated vetch only, 8.4-8.8 mg.; four cultures, wheat only, 6.0-9.1 mg.; two flasks, no plants, 5.5-5.7 mg. The variation in these results apparently does not exceed experimental error.<sup>l</sup> Extraction started with cold and finished with hot water.<sup>m</sup> Extraction with cold and hot water. Sand from three cultures only, extracted. Extraction of sand from grass-only culture secured 8.2 mg. nitrogen, and from alfalfa-only culture, 12.1 mg. nitrogen.<sup>n</sup> Cultures grown in modified coldframe.<sup>o</sup> Mean of the two cultures, accidentally put together for analysis.

TABLE 1—Continued

Exp. No.	Species and Approximate No. of Plants Used		Mineral Mixture or Solution Used	Approximate Duration of Experiment (Months)	Nitrogen Added in Seeds		N <sub>2</sub> Fixed <sup>a</sup> (mg.)	N in Non-Legumes Less That in Seeds Planted				Water <sup>a</sup> Soluble N in Sand at Close (mg.)	Remarks
	Legume	Non-Legume			Leg. Urea (mg.)	Non-Leg. Urea (mg.)		Con- trol (mg.)	Experi- mental <sup>a</sup> (mg.)	Gain or Loss by Association <sup>a</sup> (mg.)			
X	4	10-18 alfalfa	6 timothy and 3 Hungarian millet "	Thorford	3	3.00	3.89	393-516	2.1	0.5	-1.3	.....	Usual light
	4	10-18 alfalfa		3	3.09	3.94	291-348	2.1	0.8	-1.1	.....	Low light	
	1	9 sweet clover		3	2.96	6.85	864	2.1	2.2	0.1	.....	Usual light	
	1	9 sweet clover		3	2.96	5.99	372	2.1	1.8	-0.2	.....	Usual light	
	1	14 red clover		3-3	2.16	0.95	21	2.1	3.2	1.1	.....	Usual light	
	4	4 sweet pea		3-2	15.76	4.93	368-430	2.5	2.8	-8.1	.....	Usual light	
	4	4 sweet pea		3-2	15.76	4.41	193-279	2.5	2.8	-2.9	.....	Low light	
	1	4 pea (Nott's Excelsior)		2-7	55.16	4.93	363	2.5	2.15	-0.4	.....	Usual light	
	1	3 garden bean		"	54.74	4.63	85-150	2.5	0.7	-7.0	.....	Usual light	
	2	3 cowpea		"	30.00	4.63	100-114	2.5	0.4	-0.7	.....	Usual light	
	2	3 soybean		"	39.23	4.63	453	2.5	0.9	-1.6	.....	Usual light	
	1	3 soybean		"	39.23	4.41	401	2.5	0.1	-2.4	.....	Low light	
XI	1	16 Korean les- pedeza	6 timothy and 3 Hungarian millet	3-0	2.84	3.89	249	2.1	2.7	0.6	.....	Usual light	
	12	4 sweet pea	1 barley, 1 oat, 3 timothy, and 3 Italian rye grass	"	2-2	.....	.....	211 or more	.....	.....	1.7	.....	Usual light
	2	4 pea (Nott's Excelsior)	"	"	2-1	.....	.....	223-227	.....	.....	1.4	.....	Usual light
	2	3 pea (Tors- tal)	"	"	2-1	.....	.....	181-275	.....	.....	2.5	.....	Usual light
	2	4 Canada field pea	"	"	2-1	.....	.....	149-210	.....	.....	-0.2	.....	Usual light
	2	4 hairy vetch	"	"	2-1	.....	.....	261-296	.....	.....	1.2	.....	Usual light
	2	4 hairy vetch	"	"	2-1	.....	.....	.....	.....	.....	.....	.....	Usual light

<sup>a</sup> Usual light, about 2200 foot candles or 23,700 lux; low light, about 1300 foot candles or 16,100 lux; high light, about 4200 foot candles or 54,200 lux (obtained by using 1000 instead of 500 watt lamps).

at the close of those experiments where it was determined was not significantly different between experimental cultures and controls and in some cases was also negligible in amount.

In experiments II and III determinations of total nitrogen in the sand were also carried out. The results were highly variable and reflected the difficulty of making such determinations rather than any differences due to the treatments. They are therefore not included in the table.

### Discussion

It should perhaps be stated at the outset that it is not believed that these results show that VIRTANEN and others who have secured apparently opposing results are in error. It is considered that neither set of results is to be so interpreted but that some difference in conditions has caused the contrast. To date it has not been possible to determine this difference, but the results do furnish evidence concerning certain possibilities, some of which are discussed next.

GOOD GROWTH *vs.* POOR GROWTH.—In the earlier experiments the growth and nitrogen fixation by the legumes were not very satisfactory. This is the case for experiments II, III, and IV, and especially so for no. I, which is not included in the table for that reason. In the later experiments excellent growth was obtained in many cases, as well as poor growth in others. Figures 1 and 2 show an alfalfa and a soybean culture at age 35 and 33 days, respectively, and show the relative growth of legume and non-legume in the better mixed cultures. VIRTANEN'S results have usually been secured with cultures where growth was rapid; but in at least one case (17, p. 62), where the growth rate was reduced by altering the reaction of the sand, practically the same proportional excretion was obtained with the slowly growing plants as with the others.

FLUCTUATION OF CONDITIONS OF ENVIRONMENT OR OF PLANTS.—It is conceivable, although unlikely from the reports of those observing the phenomenon, that certain fluctuations in environment or internal activity of the legumes may be necessary or favorable to the excretion observed. In the work considered here several such cases of fluctuation have occurred, sometimes by design and sometimes in spite of all efforts at prevention. Among them may be mentioned the following.

(1) In experiment X the cultures were grown at the usual light intensity until it seemed evident that the result was negative and would continue so. At that time some of the cultures of alfalfa, sweet pea, sweet clover, and soybean were transferred to a compartment with about three-fourths the original light intensity. The transferred plants must have undergone an alteration in metabolic activity following the transfer. In addition, at the time of transfer, two alfalfa and two sweet pea cultures were left in practical darkness for 3 days, one culture of each being then returned to each light condition. This treatment was drastic enough to cause detectable etiolation of the portions of the stems which elongated during the period and to cause death of the developing blossom buds on the alfalfa plants. It can hardly be doubted that there was a considerable interference in the metabolism of these plants, involving a reduction in the C/N ratio.

(2) The alfalfa, sweet clover, and vetch cultures in experiment IX were clipped once or twice before the final harvest was made. The sudden reduction in photosynthesizing tissue would be expected to alter temporarily the metabolic conditions in the roots, again in the direction of a lower C/N ratio.

(3) In experiment VIII the alfalfa grew well for a time, after which a period of severe decline set in, caused principally by thrips infestation. The insects were poisoned, after which a second period of good growth occurred.

(4) In experiment II the cultures were continued until the cow-pea plants were deteriorating almost to the point where root disintegration had begun. Likewise in experiment IX some of the sweet pea plants in artificial light and all of the legumes in the outdoor cultures were deteriorating at the close.

(5) In all cultures except the sterile ones the amount of moisture in the sand fluctuated, in some cases enormously.

RELATION OF SPECIES TO RESULTS.—Suggestion has been made that the use of the same strains of green plants and bacteria used by VIRTANEN and his associates might have given the same results which they obtained. Such a possibility always exists, of course, so long as a thorough trial is not actually made. It seems unlikely, however, since (a) alfalfa, which is one of the plants employed here, has

been reported by THORNTON and NICOL (7, 9, 10) to give the result; (b) WILSON (23) has reported tests in this country in which VIRTANEN's organisms were used but with negative results; (c) mixed cultures of garden pea and grasses reported here, including in a few cases the varieties used by VIRTANEN, did not indicate that the grasses were improved by being grown in association with the pea plants; and (d) VIRTANEN has evidence (15) to indicate that alders (*Alnus* sp.) give the result as well as peas and clover, thus indicating that the phenomenon is fairly general so far as species is concerned. It is true, of course, that all workers who have reported positive results, from LIPMAN (4) to the present, have used what may be termed cool weather plants. Possibly this is due merely to the coincidence that these workers lived in a cool temperate zone, but LIPMAN did not get good results with cowpeas and soybeans, and the possibility of such a climatic correlation remains. Incidentally it may be remarked that winter wheat sowed shortly after harvesting a crop of soybeans is usually reduced in yield, owing chiefly to the removal of most of the available nitrogen from the soil by the soybeans.

INTENSITY, SPECTRAL BALANCE, AND DAILY DURATION OF LIGHT.—The experiments involved several different light intensities and durations per day. Thus the plants in experiment IV were grown for a considerable time under 12 hours of daily illumination but for the last 25 days were under 20 hours. The plants in experiment VI had about half the light intensity of those in nos. V and VII. One culture in experiment VIII was started in sunlight but was soon transferred to artificial light and 12 hours of illumination per day. The artificial light cultures of experiment IX were illuminated 12 hours per day, while those in sunlight had the illumination periods, light intensities, and spectral balance occurring naturally from April 24 to August 21. In experiment X artificial light of 18 hours' duration per day was used in two intensities. In experiment XI the duration was 18 hours per day and the intensity about twice that of the higher intensity in no. X. The one culture in experiment V which gave a possible positive result was in one of the higher light intensities, and might be considered to indicate that high light intensity will cause the excretion of nitrogen except for the fact that a com-

panion culture in the same experiment and two cultures in the same intensity in experiment VII gave negative results.

TEMPERATURE.—No study, as such, has been made of the effect of different temperatures or of fluctuations in temperature, but there have been seasonal as well as diurnal fluctuations in temperature, both of air and of sand, during the course of the work. Some experiments have been conducted at lower temperatures than others, and none has been at a fixed temperature. Except in the sterile cultures, air temperatures as a rule have been rather high, but not exceeding that of a bright summer day.

REACTION OF SAND.—No specific investigation has been made of the effect of the reaction of the sand. However, the earliest cultures were carried out at slight alkalinity and later ones at about neutrality. Further work at somewhat lower pH values is to be desired.

AERATION.—Recently VIRTANEN and his collaborators have reported (18, 20, 21) that increased aeration appears to increase the amount of nitrogen excreted. Still more recently he has reported (14) that the effect seems to be an indirect one, due to an increase in nitrogen fixed rather than to the percentage excreted. In order to determine whether additional aeration would cause the initiation of nitrogen excretion under the conditions of these studies, part of the cultures in experiment IX were subjected to extra aeration of the sand. A 7-8 cm. funnel was inverted in each treated jar in such manner that it reached to within 2 cm. of the bottom of the jar. The jar was then filled with sand, the seeds planted in the usual way, and the stem of the funnel connected with a pressure cylinder containing the air to be used. A wash bottle containing  $\text{HgCl}_2$  solution was interposed in order to moisten and incidentally to sterilize partially the flowing gas. A slow stream of air enriched to 0.5 per cent with  $\text{CO}_2$  was passed through the sand of the treated jars in the early part of the experiment; later ordinary air was used. The growth obtained and the amount of nitrogen fixed (table 1) were about the same whether the sand was aerated or not. A slight advantage for the unaerated cultures may have been due to better light, as it was necessary to place the aerated cultures permanently at one end of the bench under slightly less than average intensity. While the



added aeration did not seem to have any significant effect on the behavior of the plants, it should be remembered that the comparison involved is not between poorly aerated and well aerated cultures but between well aerated and unusually well aerated cultures, and that the results might possibly be different under the former conditions.

MOISTURE OF AIR.—The tops of the plants in most of the cultures were exposed to extremely arid conditions but with those plants grown under controlled bacterial conditions the air was much more moist. The fact that the one result which seems to be positive occurred under the latter circumstances might appear to be suggestive if it were not that the other experiments of the same kind did not produce the same result.

PROPORTION OF NON-LEGUMES TO LEGUMES.—In the beginning of an experiment the proportion of non-legumes to legumes was usually somewhat less than that used by VIRTANEN, owing partly to the use of somewhat smaller numbers of plants and partly to the more frequent use of species with small seedlings. Toward the close of an experiment it was often much less if considered on a basis of tissue mass or root surface, because the non-legumes grew very little after their seed reserves were exhausted. In view of VIRTANEN's equilibrium hypothesis (14), and particularly of his statement that an increase in the relative amount of non-legumes leads to a greater excretion from the nodules, it seems possible that an increase in the proportion of non-legumes to legumes might have led to positive results. But even if this were true it seems unlikely that any positive results so obtained would have been anywhere nearly as large as those reported by them, since the non-legumes used here showed no significant gains in practically all cases. In order to test their ability to absorb available nitrogen during the latter part of the experiments, approximately 100 mg. of nitrogen as  $\text{NH}_4\text{NO}_3$  was added to each of two of the alfalfa cultures at about the time of the second clipping in experiment IX. As was to be expected, the grass plants, although very small and unhealthy in appearance, showed in proportion to their size roughly the same ability to assimilate the added nitrogen as did the much more vigorous alfalfa plants. The evidence suggests strongly that if considerable amounts of excreted nitrogen

had been present in the sand the non-legumes would have assimilated enough of it to give results of undoubted significance in most cases.

CHARACTER OF SUBSTRATUM.—A small sample of the sand which VIRTANEN used has been received from him. As it is somewhat finer than that used in these cultures it is barely possible, in view of his statement (14) that excretion is increased by a medium of high adsorptive capacity, that the use of a finer sand or one mixed with kaolin or other colloid might have induced the result.

### Summary and conclusion

1. Mixed cultures of inoculated legumes and non-legumes have been grown in nearly nitrogen free quartz sand and minerals, and observations made on the growth, on the nitrogen accumulated by the non-legumes, and in some cases on the total and water extractable nitrogen in the sand.

2. Instead of furnishing nitrogen to the non-legumes grown in association with them, the legumes usually reduced further their already very meager growth and small nitrogen assimilation from the medium. In one case, a vetch-wheat culture, a slight benefit was noted but later experiments under similar conditions gave negative results. Likewise, in contrast to VIRTANEN's results, no excreted soluble nitrogenous matter could be extracted from the sand with water. The failure to duplicate his results does not appear to have been due to unsatisfactory growth of the plants, unchanging environmental conditions, failure to use the right species, improper intensity, spectral balance, or duration of the light, unsuitable temperature, too great acidity of the sand, or insufficient aeration of the sand. Too few data have been secured for a judgment as to the possible effect of lowering the pH to 6.5 or below, of an increase in the humidity of the air, of a considerable increase in the non-legume/legume ratio, or of the use of a medium of considerably higher adsorptive capacity; and it is possible that one of these conditions or a combination of some of them might induce the result.

3. These considerations indicate rather strongly that in the case of a considerable number of legume species, under conditions producing good growth, the heavy excretion of nitrogenous compounds observed by several workers does not always occur.

Thanks are extended to ELLEN K. RIST of this laboratory for making most of the nitrogen determinations.

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## PERSISTENT JUVENILES AMONG THE CYCADS

M. A. CHRYSLER

(WITH SIXTEEN FIGURES)

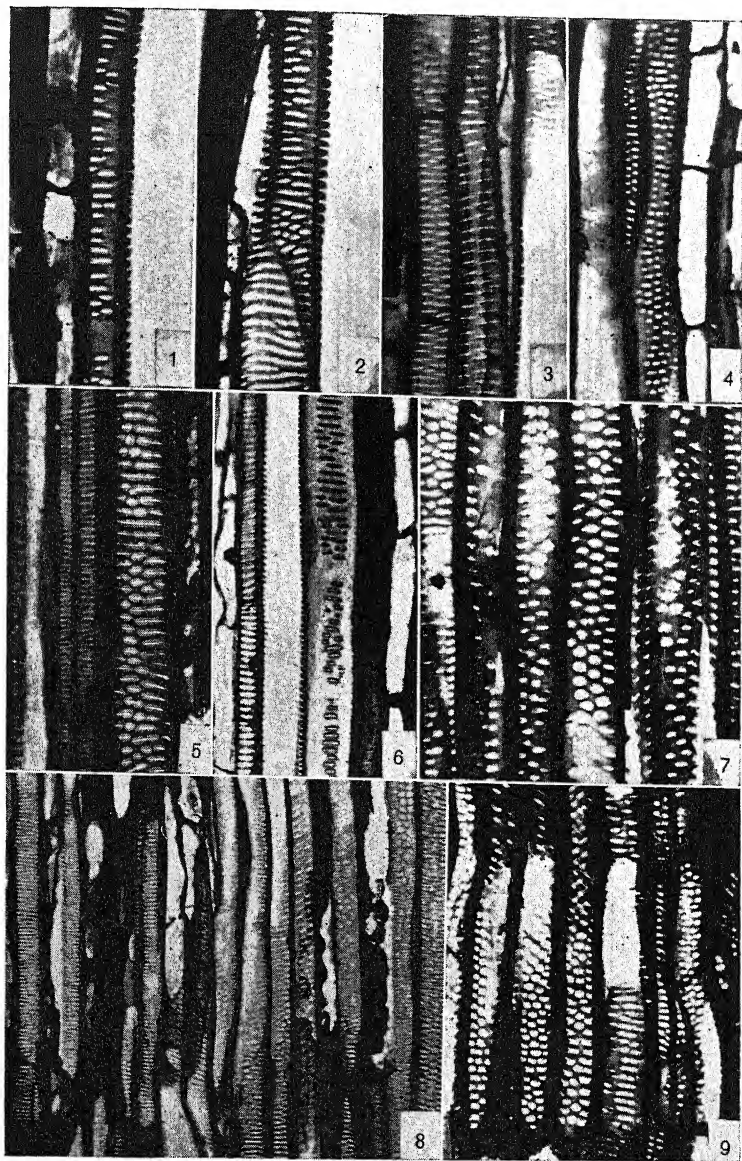
### Introduction

It has long been known that the characteristic structural element of the xylem of Cycadales is the pitted tracheid. These elements, together with parenchyma and rays, make up the bulk of the wood in the stem in seven out of the nine genera. *Zamia* and *Stangeria* alone show in their stems a xylem consisting of scalariform tracheids, together with the usual parenchyma. In all genera which have been examined, the tracheids near the pith present scalariform or reticulate markings, and in some genera, for example, *Microcycas* (3), there is a gradual transition from the scalariform to the pitted condition, until finally the latter becomes established.

Suspecting that the reports on the scalariform condition in *Zamia* might be due to examination of relatively immature plants, the writer secured from Florida a plant of *Z. floridana* having a caudex measuring 130 mm. in diameter at its greatest width, and bearing five megasporangiate cones. Sections of this stem showed none but scalariform tracheids, except near the pith, where tight spirals and reticulate thickenings were in evidence.

A little later a study of leaf bundles of this species was undertaken, and BAILEY's report (1) of transitional and pitted tracheids in the centripetal xylem of the rachis was confirmed (figs. 1-3). Since the leaf is regarded as a conservative organ compared with the stem, the presence of the advanced condition of bordered pits appears surprising if not disconcerting from a morphological standpoint—the leaf ought to be more primitive than the stem, rather than less primitive. Is the doctrine ("canon" is JEFFREY's 5 word) of conservative organs discredited by these observations?

The answer to this question has been furnished by a study of species of *Zamia* possessing a trunk. All of the species native to Florida and the West Indies have a tuberous stem or caudex, and



FIGS. 1-9.—Fig. 1, *Zamia floridana*, pitted tracheid from centripetal xylem of leaf rachis. Fig. 2, same, leaf rachis, ends of two tracheids showing transitional stages. Fig. 3, same, leaf rachis. Contact between tracheids and parenchyma, showing half-bordered pits. Fig. 4, *Z. kickxii*, leaf rachis; pitted tracheids. Fig. 5, *Z. furfuracea*, leaf rachis; transitional stages. Fig. 6, *Z. pseudoparasitica*, leaf rachis; spiral and pitted tracheids. Fig. 7, same, outer region of xylem of stem showing multiseriate bordered pits. Fig. 8, same, inner region of xylem of stem showing scalariform, transitional, and pitted tracheids. Fig. 9, *Z. tuerckheimii*, outer region of xylem of cone axis showing transitional and pitted tracheids.

anatomical studies have apparently been restricted to these. In Central America, however, there are several representatives of the genus possessing well defined trunks. Through the generous cooperation of Drs. A. SKUTCH and W. R. HATCH, material of trunk-forming species has been collected in Costa Rica and Guatemala. An examination of this material has disclosed entirely normal pitted tracheids in the stem wood (fig. 7), just as occurs in other trunk-forming genera such as *Dioon* (2).

Before proceeding to a description of the new material, it may be pointed out that the occurrence of pitted tracheids in the trunk wood of these species at once explains the occurrence of such elements in the petioles of the various species, including those with a tuberous stem. In this respect the petiole is neither more nor less primitive than the stem, whatever other conservative features the petiole may show. It would appear that the tuberous *Zamia* species have petioles whose xylem shows pits because their ancestors were treelike and had a xylem like that of the other genera of cycads.

### Observations

#### STRUCTURE OF TRUNKS

The material provided by Dr. SKUTCH has been identified as *Z. pseudoparasitica* Yates, collected in heavy forest, Prov. San José, Costa Rica, at an altitude of 1070 m. It is reported to have a trunk up to 1 m. high, with leaves almost 2 m. long. The pieces of stem used in this work are 70 mm. in diameter, and the xylem ring measures 3-4 mm. in thickness. The general features of this layer are entirely typical of cycads such as *Dioon*, consisting of wide tracheids with a sprinkling of parenchyma cells, and rays of varying width. For a thickness of about forty tracheids nearest the phloem, the uniform pattern of thickening is circular bordered pits, as is represented in figure 7. Toward the pith, however, the markings are transitional (with slitlike and partly scalariform markings), and finally scalariform and scalariform-reticulate (fig. 8).

Dr. HATCH's material includes two species, the first of which has been identified as *Z. skinneri* Warsc. It was found on "limestone outcrops, under a rather dense canopy in montane or lowland rain-forest," on Rio Chiacte, Guatemala, altitude 1300 m. The trunk is

described as prostrate and sinuous for 1 m., then erect for 1.2 m., width tapering from 120 mm. at the apex to 50 mm. at the soil line. The block which has been cut measures 75 mm. in width, and has a xylem ring 2 mm. thick. As in the preceding species, the inner region of the xylem shows scalariform tracheids, while the outer region presents a truly remarkable appearance on account of the tertiary rings or spirals which are plentifully laid down upon the pitted walls. As may be seen from figure 13, the pits are circular, bordered, and closely disposed, while the rings are stout and conspicuous. In some cases the rings lie over pits, while in others they lie between pits. Scalariform and reticulate walls as well as pitted ones may be reinforced by these thick rings. Tertiary thickenings on the walls of tracheids in cycads have been mentioned by SIFTON (8). In the absence of illustrations, it is difficult to determine whether SIFTON referred to such extremely stout annular thickenings as are so abundant in these trunk-forming *Zamia* species.

The other specimen from Guatemala, *Z. tuerckheimii* Donn. Smith, was collected in the same habitat as *Z. skinneri*, at Alta Verapaz, and had the same habit of growth, being prostrate for nearly a meter, then erect for 0.4 m. The pieces under study have a diameter of 50-90 mm., and the xylem is 1 mm. thick, showing a series of only about twenty-five tracheids, all of which have scalariform thickening. The considerable length of this trunk, the exceedingly thin vascular ring, and the high rainfall of the habitat (average of seven years is reported as 178 inches annually) indicate that this plant grew rapidly and is a comparatively young specimen. It bore a single cone while in the case of *Z. skinneri* cones were found in abundance, the micro-cones in groups of 2-6 on a single plant. If we are correct in regarding the specimen of *Z. tuerckheimii* as a young plant, it is easy to understand why it shows no pitted tracheids when we recall the writer's observation (3) that *Microcycas*, a rather slow-growing species with well developed trunk, shows a xylem ring with series of forty scalariform or transitional tracheids before the adult condition is established. In this connection, it may be recorded that the caudex of *Z. floridana* having diameter 130 mm., previously mentioned, shows a xylem ring 2.5 mm. in thickness, with approximately seventy-five tracheids in radial series, all scalariform with the exception of the protoxylem, which as usual is scalariform-reticulate.



One other case of a *Zamia* with trunk has been examined. In a letter to the writer, Dr. C. J. CHAMBERLAIN reports that in *Z. monticola*, a species with a stout trunk, he has observed bordered pits. This observation is entirely in accord with the writer's studies.

#### STRUCTURE OF PETIOLE

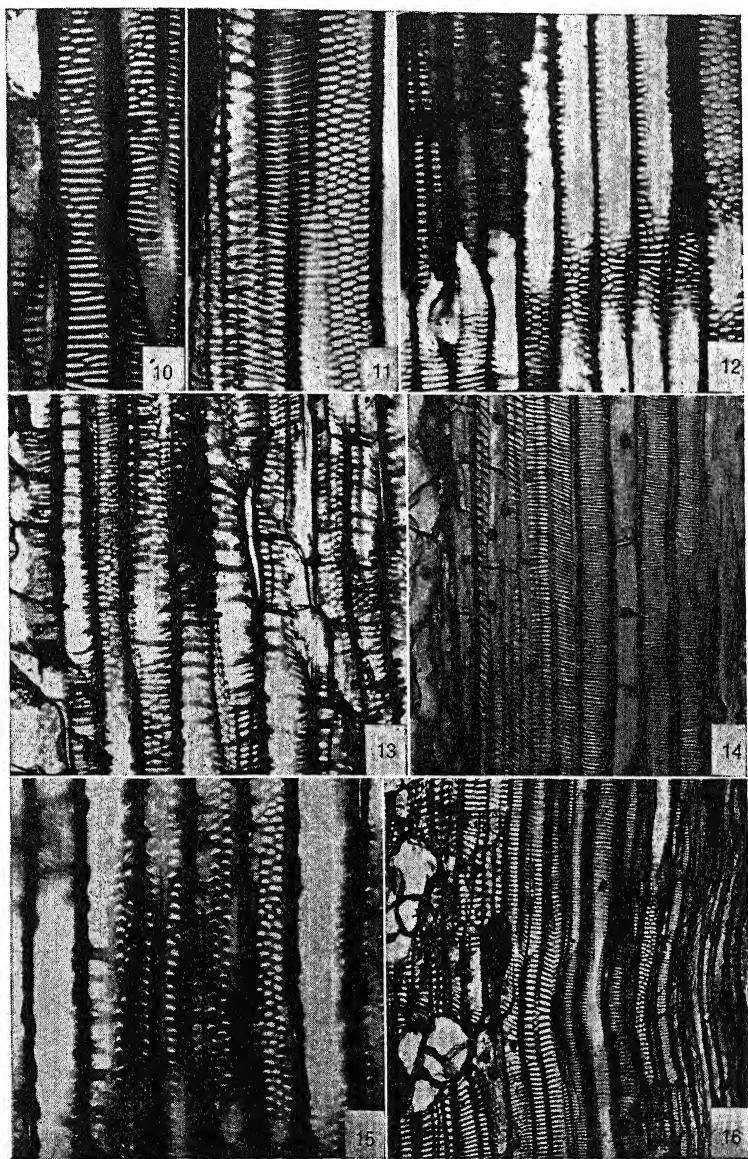
The observations on the tracheids of the petiole in *Z. floridana* have been extended to as many of the species of *Zamia* as have been available, namely, *umbrosa* Small, from Daytona, Florida; *latifoliolata* Prenl. and *kickxii* Miq., from Cuba; *furfuracea* Ait. (a Mexican species presented by the Director of the Cuba Experiment Station); *media* Jacq. (probably) from Puerto Rico through the kindness of Dr. M. T. Cook; an undetermined species from Hope Gardens, Jamaica; and the three treelike species from Central America. In each of these species the centripetal xylem shows tracheids ranging from spiral through scalariform to circular pitted, with elements intermediate between the last two. The pits are generally not in the numerous crowded series characteristic of genera such as *Ceratozamia* (see figure in BAILEY 1), but the circular pits are apt to occur mixed with slit-shaped ones and true scalariform openings on the same tracheid. Typical appearances are shown in the photographs of *Z. kickxii* (fig. 4), *furfuracea* (fig. 5), and *pseudoparasitica* (fig. 6). In many of the bundles, tracheids showing a condition beyond the transitional stage are scarce. It is of interest to note that the petiolar bundles of *Z. tuerckheimii*, a trunk-producing species, show well marked circular pits in some of the tracheids, although the one available stem presents only scalariform tracheids (*vide supra*).

The petiolar bundles of *Z. skinneri* present the further peculiarity of having ring-shaped thickenings as well as circular pits on some of the tracheids. This condition has already been noted as characteristic of the stem of this species, so that it appears to be a more or less specific feature, not restricted to *Z. skinneri* however.

Attention may be called to the abundance of pits on walls separating a tracheid from a parenchyma cell, as is shown in figure 3. The pits in this case are of course half-bordered.

#### PETIOLAR BUNDLES OF STANGERIA

The occurrence of circular pits in the leaf of *Zamia* spp. suggests an examination of *Stangeria*, the other genus having stem wood made



FIGS. 10-16.—Fig. 10, *Stangeria paradoxa*, transitional tracheids from centripetal xylem of leaf rachis. Fig. 11, same, spiral, scalariform, and pitted tracheids from leaf rachis. Fig. 12, *Zamia floridana*, secondary xylem of root showing transitional and pitted tracheids. Fig. 13, *Z. skinneri*, xylem of stem showing tertiary rings or spirals on pitted tracheids. Fig. 14, same, entire width of xylem of cone axis, with spiral and scalariform tracheids. Fig. 15, *Z. pseudoparasitica* showing xylem typical of root, with conspicuous tertiary spiral or ring-shaped thickening as well as bordered pits. Fig. 16, *Cycas revoluta*, xylem of cone axis with protoxylem at left, and pitted tracheid near phloem on right.

up of scalariform tracheids. Material has been available through the courtesy of the Director of the New York Botanical Garden. An average condition is shown in figure 10 and a more unusual one in figure 11, in which it is easy to make out the pitted condition, as well as transitional stages. Scalariform and spiral elements also occur toward the region of the protoxylem. Pits are most frequent in the outer or mature regions of the xylem mass, including the edges or flanks of a bundle; they are also abundant where a tracheid comes into contact with a row of parenchyma cells (*cf. Z. floridana*, fig. 3). These observations are at variance with the statement of MARSH (6), "no trace of ordinary bordered pitting was found anywhere in the plant." In his description of the petiolar bundles he states, "the metaxylem had pitting of a close scalariform type." This discrepancy may probably be due to the lack of uniformity in structure of different regions of the long rachis of the leaf in *Stangeria*.

#### BUNDLES OF CONE AXIS

SCOTT (7) showed that the peduncle of certain cycads possesses centripetal xylem, hence the reproductive axis has been regarded as a conservative organ. The xylem of the cone axis ought to throw light on the anomalous xylem of the tuberous species of *Zamia*. Cone axes of the following species have been available for study: *floridana*, *kickxii*, *media* (?), *pseudoparasitica*, *skinneri*, and an undetermined species (probably *gutierrezii*) under cultivation in Cuba by Mr. H. A. van Hermann. In nearly all cases the bundles show a particularly clear series, beginning with narrow spiral or annular tracheids of the protoxylem (endarch), followed by wider spirals, scalariform tracheids, and finally phloem. This condition is illustrated in figure 14 for *Z. skinneri*. There is here no disturbing event such as the disruption of the primary xylem by growth of intruding parenchyma cells, characteristic of the stem, hence the recapitulation series is clear cut, although brief and incomplete on account of the absence of tracheids showing circular pits.

But the single cone axis of the Guatemalan *Z. tuerckheimii* which has been available shows the exceptional appearance of a series of well developed pitted and transition tracheids (fig. 9). This feature occurs in the peduncle, which appears aged or possibly wounded,

judging from the unusually dark color of the cell walls. The tracheids, moreover, are not so straight as usual, but appear more or less undulating. On account of the scarcity of material, it would be rash to do more than venture the suggestion that we may here see a traumatic reversion.

It has appeared desirable to extend the observations on peduncles to the other genera. In order to do this it has been necessary to enlist the services of the following, whose assistance in securing material of cones is most gratefully acknowledged: Dr. W. H. BROWN, Philippine Bureau of Science (*Cycas circinalis*, *C. rumphii*); Dr. C. J. CHAMBERLAIN, University of Chicago (*Dioon*); the late Dr. A. A. LAWSON, Sydney, N.S.W. (*Bowenia*, *Macrozamia*); Golden Gate Park, San Francisco (*Ceratozamia*, *Encephalartos*); Horticultural Park, Philadelphia (*Ceratozamia*, *Encephalartos*); New York Botanical Garden (*Stangeria*); Reasoner Brothers, Oneco, Florida (*Cycas revoluta*); Shaw Gardens, St. Louis (*Cycas circinalis*).

The results of this survey indicate that, so far as my material goes, in the genera *Dioon* (*edule*, *spinulosum*), *Ceratozamia*, *Encephalartos* (represented by *villosus*), *Macrozamia*, and *Microcycas* the condition is the same as that figured for *Zamia skinneri*, that is, the bundles of the peduncle show a series of narrow spiral, wide spiral, and scalariform tracheids, but nothing more advanced.

In *Stangeria*, however, the scalariform pits of the tracheids nearest the phloem show a tendency to become divided, thus constituting what we have called the transitional stage. Certain of the tracheids of this region, especially on the flanks of the bundles, show a reticulate appearance without distinct border to the meshes; but actual circular pits are practically absent in my material.

The cone axis of *Cycas revoluta* (microsporangiate, of course) goes a step further, having numerous tracheids with transitional markings and a few next the phloem with definite circular multiseriate bordered pits (fig. 16), thus exhibiting the complete recapitulation.

Finally, the cone axis of *Bowenia serrulata* shows numerous rows of circular pits, as well as slit-shaped pits and transitional stages. This observation is interesting on account of the tuberous habit of the stem in this genus.

That different species of a genus may vary in the extent of de-

velopment of the xylem is shown by the observation that in the cone axis of *Cycas circinalis* pits appear to be absent.

The incomplete recapitulation in the cone axis of many if not most of the cycads is not surprising in view of the short life of this organ, compared with the stem. The cone axis is continuous with the stem axis, and generally lays down a moderate thickness of secondary growth, say from fifteen to twenty-five rows of tracheids; but after the cone has begun to decay the cambium in the stem continues to produce tracheids, eventually reaching the condition of circular bordered pits. In fact, the only way in which a cone axis may produce pits is by hastening its recapitulation, and this appears to have taken place in *Bowenia*.

#### STRUCTURE OF ROOT

An examination of the root of *Zamia floridana* shows a structure surprisingly different from that of the stem. Instead of the uniform series of scalariform tracheids, the root presents an abundance of elements with circular and elongated pits, with many of the transitional elements (fig. 12). Any of these tracheids may be further strengthened by rather broad rings or spirals which may or may not show a definite tertiary origin. The appearance is hence much like that of the stem of the dendritic *Z. skinneri* (fig. 13).

The secondary xylem of the root of *Z. pseudoparasitica* (fig. 15) consists almost entirely of broad tracheids whose walls are covered with circular bordered pits, generally reinforced by the annular thickenings already noted as occurring in the root of *Z. floridana* and the stem of *Z. skinneri* (roots of the latter have not been available).

With respect to the other genera, roots of *Stangeria* have generously been supplied through the courtesy of the Director of the New York Botanical Garden. Although some of these roots have a diameter of 10 mm., their central cylinder shows but slight development, having only about sixteen rows of tracheids in the secondary xylem, all showing scalariform thickening. Apparently these roots are not so old as their diameter would indicate, and it yet remains to be seen whether an older root would show circular pits.

The genera having circular pits in their stems might be expected

to show them also in their roots. I have been able to check this point by examining roots of *Bowenia serrulata*, *Ceratozamia*, *Dioon spinulosum*, *Encephalartos villosus*, *Macrozamia denisonii*, and *Microcycas*. It is found that all of these show abundance of pits.

### Discussion

The genus *Zamia* falls into line with the other genera with regard to the structure of its vascular elements. The scalariform condition, generally regarded as distinguishing this genus, appears to be restricted to the tuberous species. A survey of the various organs shows that circular pits are characteristic not only of the stem (in the trunk-forming species) but also of the root, leaf, and even of the cone axis in exceptional cases. It is impossible to avoid associating the scalariform tracheid with the tuberous habit, whether a complete survey of the genus would bear out this assumption or not. To reach a conclusion of such general import would involve as well a study of a number of members of the genera *Macrozamia* and *Encephalartos*, which share with *Zamia* the inclusion of tuberous as well as trunk-forming species. The requisite material is not now available. The only case which can be recorded at present is based on material kindly provided by Dr. C. J. CHAMBERLAIN. The stem of *Encephalartos altensteinii*, which "sometimes reaches a height of ten feet" (CHAMBERLAIN), shows an abundance of circular pits in its tracheids; while *E. brachyphyllus*, a strictly tuberous species from Zululand, shows nothing more advanced than scalariform markings.

It is submitted that the evidence so far accumulated points to the inference that the tuberous species of *Zamia* have literally never "grown up," that is, they are persistent juveniles in habit and in some of their vascular tissues. That they are not completely immature is obvious when one recalls the group of strobili borne by an average plant of *Z. floridana*. The ancestors of the modern *Zamia* species may be pictured as trees on account of the tropical range of the family. Perhaps the tuberous species represent members of the genus that have met evil days in respect of climate, or an extension of the genus into less favorable climatic regions; *Z. floridana* lives in calcareous sand, freely exposed to the sun, while *Z. kickxii* of western Cuba has its caudex buried in exceedingly hard and unfriend-

ly soil. On the other hand, the trunk-forming species of Central America are reported to occur in montane or coastal rain forest. Moreover the stiff thick leaflets of these tuberous species are in marked contrast to the broad and thinner leaflets of *Z. pseudoparasitica*. But it would be unwise to press this point too far, as measurements of leaf thickness in a number of species show. And it will be recalled that some trunk-forming cycads (for example, *Dioon*) live in an exceedingly dry habitat. WIELAND (9) has recorded his mature opinion that the cycadeoids of the Cretaceous, which at best have a short trunk, represent a climax of xerophytic plants.

But for one reason or another, the tuberous species of *Zamia* retain a juvenile habit and xylem. There are other better known plants which are persistently juvenile in habit, for instance the so-called *Retinispora* species, which are regarded as members of the genus *Chamaecyparis* or *Thuja*. These ornamental conifers retain the juvenile foliage for an indefinite period and are usually sterile. Occasionally, however, seed cones are produced, as has been observed in New Brunswick during 1936 in the case of *R. pisifera*. In the Royal Gardens at Kew is a group of large specimens of a juvenile form of *Cryptomeria*. The seedling foliage of *Thuja* is a familiar object in northern swamps, and the mixture of juvenile and mature branches of *Juniperus virginiana* will also be recalled. COCKAYNE (4) remarks that in New Zealand no fewer than 200 species of spermatophytes pass through a juvenile stage. This is especially well marked in *Dacrydium* and *Podocarpus*, and may persist for as long as eighty years in *P. dacrydioides*.

The monotypic *Stangeria* appears to illustrate the same juvenile features as *Zamia* spp. This is a particularly interesting case on account of the primitive features shown by the leaf. It is a matter of common knowledge that when the plant was first grown at Kew it was mistaken for a fern. But the xylem of the stem is not primitive, it is immature, or in other words its development has been arrested, as is indicated by the occurrence of pitted tracheids in the leaf and cone axis.

It would be indeed strange if the xylem of present day cycads were not pitted, in view of the fact that many Paleozoic plants (for example, *Lyginopteris* and *Cordaitea*) had at that early period reached

the stage of a tracheid with crowded circular pits. Longitudinal sections through the stem of *Lyginopteris* show circular pits in the mesarch leaf traces as well as the central cylinder of the stem. Somewhere among these plants must be the ancestors of our modern cycads.

The present observations on the tracheids of cycads are summarized in table 1. The prevalence of circular pits in the group is obvi-

TABLE 1  
NATURE OF PITTING IN XYLEM OF CYCADS, REPRESENTING  
MOST ADVANCED CONDITION REACHED

MATERIAL	HABIT	STEM	ROOT	PETIOLE	CONE AXIS
<i>Bowenia serrulata</i> .....	Tuber	Pitted	Slit-shaped	Pitted	Pitted
<i>Ceratozamia mexicana</i> ...	Trunk	Pitted	Pitted	Pitted	Scalariform
<i>Cycas revoluta</i> .....	Trunk	Pitted	Pitted	Pitted	Scalariform and transitional
<i>Cycas circinalis</i> .....	Trunk	Pitted	Pitted	Pitted	Scalariform
<i>Dioon spinulosum</i> .....	Trunk	Pitted	Pitted	Pitted	Scalariform
<i>Encephalartos villosus</i> ...	?	?	Pitted	Pitted	Scalariform
<i>Macrozamia spiralis</i> ....	?	Pitted	?	Pitted	Scalariform
<i>Macrozamia denisonii</i> ....	Trunk	?	Pitted	?	?
<i>Microcycas</i> .....	Trunk	Pitted	Pitted	Transitional	Scalariform
<i>Stangeria</i> .....	Tuber	Scalariform	Scalariform?	Pitted	Transitional
<i>Zamia floridana</i> .....	Tuber	Scalariform	Pitted and rings	Pitted	Scalariform
<i>Zamia kickxii</i> .....	Tuber	Scalariform	?	Pitted	Scalariform
<i>Zamia pseudoparasitica</i> ..	Trunk	Pitted	Pitted and rings	Pitted	Scalariform
<i>Zamia skinneri</i> .....	Trunk	Pitted and rings	?	Pitted	Scalariform

ous. Their relative scarcity in the cone axis also is noticeable. The differences shown by the xylem of the various organs may be regarded as so many cases of recapitulation of the phylogenetic development of the tracheid from steep spiral to circular pitted.

STEM.—In most members of the group there is a complete and gradual recapitulation in the stem. For instance, it has been observed (3) that in *Microcycas* a series of approximately forty tracheids is formed before the pitted condition is fully established, and this number does not include the protoxylem which in the stem is



disrupted by intrusion of parenchyma. In *Stangeria* and the tuberous *Zamia* spp., however, the ontogeny is incomplete or arrested as well as gradual.

CONE AXIS.—In most genera the cone axis also illustrates an incomplete recapitulation which, however, is regular on account of a lack of disturbance in the region of the primary xylem. Some genera show a complete recapitulation, for example, *Bowenia*, which is nevertheless a genus with tuberous stem. In all cases which have been observed, the stages between steep spiral and scalariform are passed over rapidly, in contrast with the deliberate habit of the stem. The case of *Cycas* is of special interest. We have become accustomed to think of this as a particularly primitive genus, a "living fossil," on account of the fact that its megasporophylls are not aggregated into a cone but are distinct and leaflike. But the microsporophylls show no such primitive features, either external or internal, for the xylem illustrates a complete recapitulation and has little or no sign of centripetal growth. Perhaps, however, we should regard a complete recapitulation as nearer the ancestral condition than is the incomplete one. (A more extended treatment of this case is in preparation.)

ROOT.—The root appears to pass rapidly through the early phylogenetic stages, for the secondary xylem in most of the genera so far examined consists of pitted or transitional tracheids. It might be expected that in an organ so shielded from external factors of an extreme nature the primitive features would persist, but such does not seem to be the case. At all events the abundant pitted tracheids of the root repeat the condition characteristic of what are believed to be the ancestors of the cycads.

LEAF.—The leaf differs from the other organs in the almost complete replacement (except at the base of the petiole) of the usual centrifugal by a group of centripetal tracheids. In all of the genera this group exhibits a rapid progression from normal protoxylem to pitted metaxylem, that is, the recapitulation in most genera is complete but condensed. In *Stangeria* and most species of *Zamia*, however, the series hardly passes beyond the transitional stage, although circular bordered pits have been shown to occur. Since all of the centripetal xylem is primary, the completeness of the recapitula-

tion is worthy of remark. In none of the cycads can be observed that overlapping or telescoping of primitive and advanced elements which BAILEY (1) has described for conifers.

### Summary

1. An explanation is offered for the apparently anomalous composition of the xylem of the stem in *Zamia* and *Stangeria*.
2. It is shown that although the stem wood of tuberous species of *Zamia* does not pass beyond the scalariform stage, certain trunk-producing species from Central America show abundance of tracheids with circular bordered pits.
3. The presence of circular pits in the tracheids of the leaf rachis is hence explained; otherwise the occurrence of pits would constitute an exception to the doctrine of conservative organs.
4. The tuberous species of *Zamia* are regarded as persistent juveniles with respect to their growth habit and their xylem; that is, they remain immature vegetatively although they reproduce freely.
5. A comparison is made between these juvenile cycads and such persistent juveniles as the *Retinispora* species and other conifers.
6. A similar explanation is considered to apply to *Stangeria*.
7. All organs of the cycad plant are surveyed in order to test the validity of the explanation, and the differences shown by the xylem in stem, cone axis, root, and leaf are interpreted in terms of relative completeness and rapidity of the recapitulation which the different organs exhibit in the various genera. The stem exhibits a complete although gradual recapitulation, incomplete, however, in the tuberous species of *Zamia*; the reproductive axis has a much more condensed series, generally incomplete; the root quickly passes through the early phylogenetic stages; finally the leaf shows in its centripetal xylem a much abbreviated recapitulation, which tends to be incomplete in the tuberous members of the group.

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SEEDLING ANATOMY OF *CYNARA SCOLYMUS*  
CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 479

WALTER S. PHILLIPS  
(WITH TWENTY-TWO FIGURES)

Introduction

According to DE CANDOLLE (2) *Cynara scolymus* L., the Globe artichoke, is a native of the Mediterranean region. MORIS, DE CANDOLLE reports, cultivated *C. cardunculus* and *C. scolymus* side by side and found no true specific distinctions. He therefore applied the name *C. cardunculus* var. *sativa* to *C. scolymus*. STURTEVANT (14) discussed the synonymy and history of this species. At the present time the most important area for commercial production in the United States is along the California coast, between Santa Barbara and San Francisco, where about 9000 acres are devoted to its cultivation (11).

Little morphological work has been done with *Cynara scolymus*, and this has dealt chiefly with the chemical contents of the flower heads, which constitute the commercial part of the plant. OKEY and WILLIAMS (9) reported inulin as the chief carbohydrate, with a small percentage of reducing sugars also present. SCOTT (11) discussed propagation briefly and also included chemical analyses of the subterranean and aerial parts of the plant. LE FEUVRE (8) and WELLINGTON (19) investigated propagation and cultivation. JEFFREY (6) considered the tribe Cynareae as "transitional anatomically from the Tubuliflorae to the Liguliflorae," and regarded the oil canals found in *C. scolymus* as primitive structures in what he considered to be the more stable parts of the plant. SACHS (10) and VAN TIEGHEM (18) described the oil canals or ducts, which are of schizogenous origin and occur between the two layers of the endodermis. These have been reported as occurring in many members of this family, according to SOLEREDER (13).

Material and methods

The seeds used in this investigation were obtained from the Bureau of Foreign Plant Introduction at their Chico, California, sta-

tion, and also from a commercial seed company. Three varieties, Green Globe, Flat Brittany, and Large Globe or Paris Improved, were found to be practically identical in their seedling anatomy. The straightest seedlings were grown on moist paper in tumblers, but in order to check their development with seedlings grown in soil, akenes were also planted in soil and serial sections of these plants were compared with those germinated on paper. No morphological differences were found.

Navashin's solution and chrom-acetic solutions proved the best fixatives. After fixing, the material was washed for several hours in warm water to soften and dissolve the inulin as much as possible. Serial sections were cut 8-15  $\mu$  in thickness and stained with Flemming's triple stain or with safranin and fast green. The safranin-fast green stain was very satisfactory in making drawings with micro-projection apparatus.

### Investigation

*Cynara scolymus* is propagated by suckers or by seed. The sucker method is usually followed because it brings the plants into commercial production sooner. The plant is a perennial with the rosette habit, the flower stalk not appearing until the second year in plants grown from seed. As soon as the flower stalk dies back, about a dozen new shoots develop from axillary buds of the rosette leaves. SCOTT reports that these new shoots develop an adventitious root system and that the old primary root becomes fleshy and functions as a storage organ.

The fruit is an akene containing a single erect anatropous ovule. Endosperm is lacking and the nucellar tissue consists of a thin membranaceous layer of cells closely adhering to the ovary wall. The small hypocotyl and epicotyl lie at the basal end of the ovary, and the two thick cotyledons fill the remainder.

At germination the primary root pushes through the coats of the akene by the second or third day. Rapid elongation of the primary root takes place first; then the hypocotyl elongates and raises the cotyledons, partially inclosed in the akene, above the ground. The cotyledons expand rapidly and function as fleshy photosynthetic

leaves for several days before the first epicotyledonary leaves appear. The mature cotyledons are obovate, tapering slightly toward the thick fleshy petioles.

#### PRIMARY ROOT

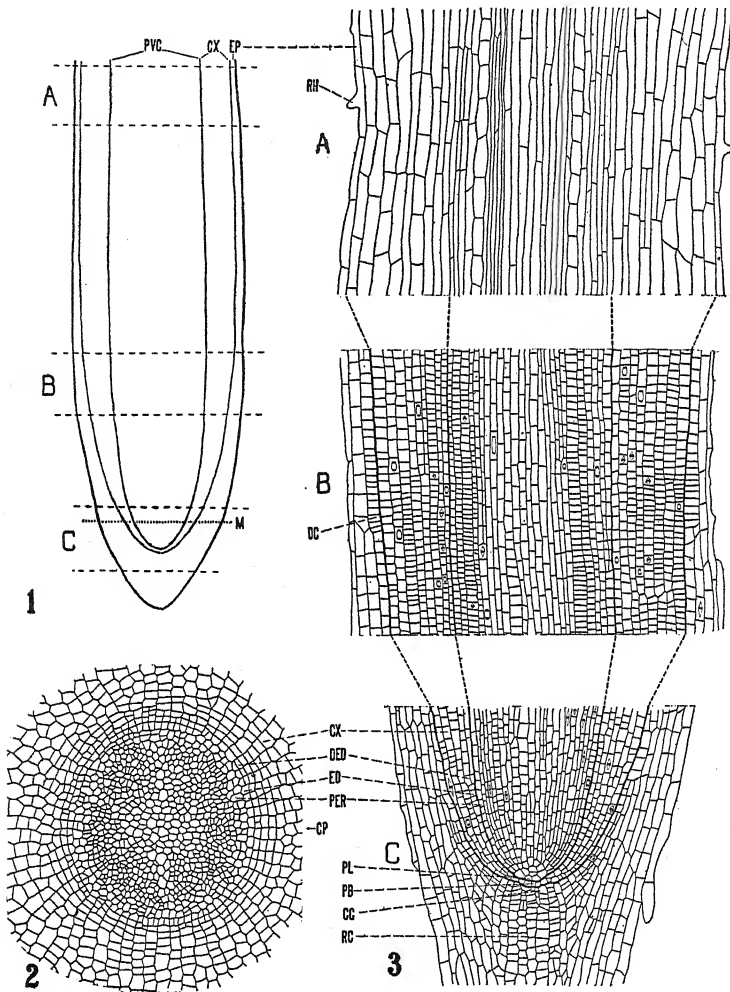
The primary root is diarch, with the four or five protoxylem elements of each point alternating with two lateral bands of phloem cells. JEFFREY (6) has figured the primary root as triarch, but in the seedlings examined this condition was never encountered. Some of the adventitious roots of older plants were tetrarch. Parenchymatous cells separate the phloem from the xylem in the region where the cambium is later to develop, and there are several large parenchymatous cells in the central region of the stele. This whole cylinder of vascular tissue is surrounded by the pericycle, which is at first uniseriate. The primary xylem elements remain intact after secondary thickening has taken place, and are clearly discernible in the mature root.

The endodermis limits the cortex centripetally, but Casparian strips are not developed until after there has been some secondary thickening. The endodermis can be distinguished from the other cortical cells by the early development of primary endodermal oil ducts, which accurately locate the endodermal layers even before the formation of the Casparian strips. The layers of cortical cells adjacent to the endodermis have a radial arrangement which is lost in the outer limits of the cortex. The cortex persists intact for a considerable time, but after secondary thickening of the axis has started, a phellogen is formed midway between the endodermis and the epidermis and the outer cortical cells and epidermis slough off.

The development of the primary root axis is similar to that described by JANCZEWSKI (5) for *Helianthus annuus*. It follows his third type, in which plerome and periblem produce stele and cortex respectively and a common layer initiates both the epidermis and calyptra.

This common layer of cells overlying the periblem has been termed the calyptrogen-dermatogen by CROOKS (3) because both the root cap and the epidermis are derived from it. ERIKSSON (4) has referred to it as the dermacalyptrogen. This layer divides in a series of periclinal divisions at the apex of the root, giving rise to the more

or less radially arranged cells of the terminal portion of the root cap (figs. 1, 3C). Periclinal divisions also occur in the lateral por-



FIGS. 1-3.—Fig. 1, diagrammatic representation of root tip. A, B, and C represent areas shown in detail in fig. 3. M, position of cross section shown in fig. 2. Fig. 2, cross section through meristem of root at level where endodermal layer divides. Fig. 3, A, B, C, longitudinal sections of root. *cg*, calyptrogen; *cp*, cotyledonary plane; *cx*, cortex; *ded*, double endodermis; *dg*, dermatogen; *ed*, single endodermal layer; *ep*, epidermis; *pb*, periblem; *per*, pericycle; *pl*, plerome; *rc*, root cap; *rh*, root hair.

tions of this layer, where anticlinal divisions are taking place. In this manner the root cap becomes an elongate structure extending some distance upward from the tip of the root, but being thickest at the apex. The lateral portions of this layer form the epidermis by a series of anticlinal divisions (fig. 3*B*).

The cortex is derived from a single layer of cells, the periblem, which lies directly inside the calyptragen-dermatogen layer. The endodermis, which limits the cortex on the inside, is a cylindrical layer of tissue two cells in thickness, with structural features unlike those of the cells on either side. This double layer of cells originates from the innermost layer of the periblem near the apex of the root by a single periclinal division (figs. 2, 3*C*). The cytoplasm in the endodermal cells is dense and contains no inulin crystals. Oil ducts develop by the formation of schizogenous intercellular spaces between these two layers of endodermal cells. These are not formed where the endodermis lies exterior to the protoxylem points. BARY (1) considers corresponding cell layers in other plants as endodermal but in this case only the layer next to the stele has been found to develop Casparian strips. These ducts have been described by SACHS (10) and VAN TIEGHEM (18). SOLEREDER (13) summarizes the work on oil ducts done in this and other groups of plants.

The plerome gives rise to the stele and consists of a small group of cells directly within the periblem. The larger parenchymatous cells at the center of the axis are discernible at an early stage in the differentiation of the stele. The pericycle, which is at first a single layer, becomes several cells thick by means of tangential divisions of its cells (fig. 21). The first vascular tissue to differentiate is the primary phloem, which is formed by divisions in a vertical plane of the plerome cells immediately within the pericycle. Six primary phloem ducts, three on either side of the cotyledonary plane, are at first evident at the outside limits of the phloem, but these are soon crushed by the enlargement of cells which differentiate as sieve tubes and companion cells. The primary xylem is differentiated centripetally. The protoxylem elements have annular-spiral thickenings, and the metaxylem scalariform thickenings.



## PROVASCULAR SEEDLING ANATOMY

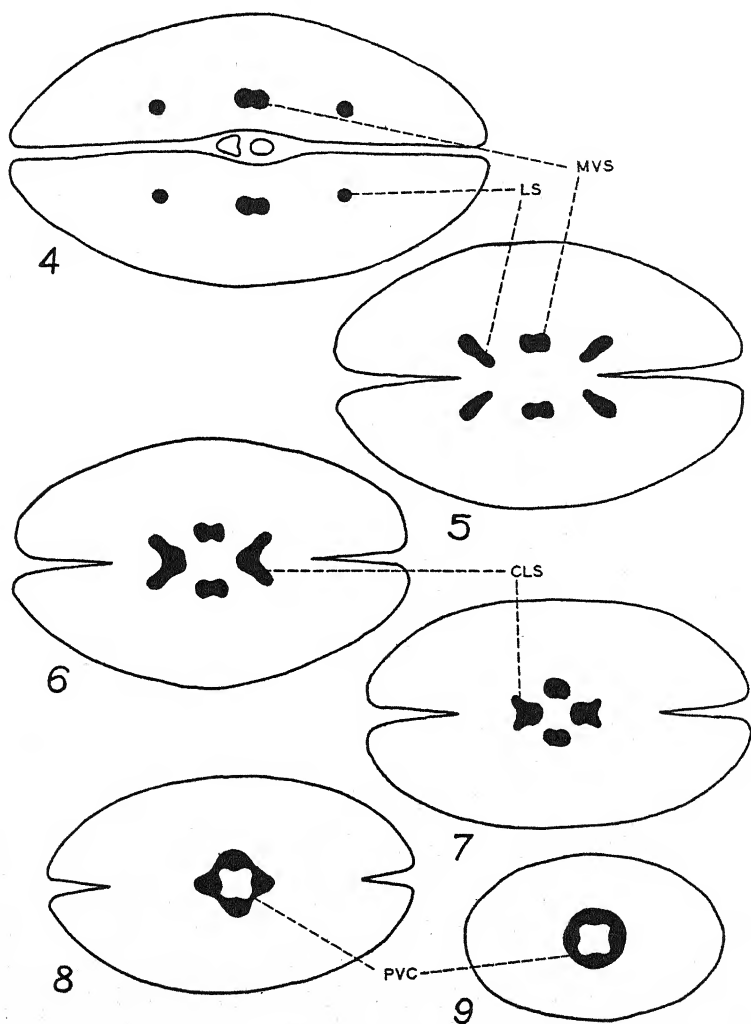
In a day-old seedling the provascular strands can easily be traced and the ultimate arrangement of the vascular system is clearly indicated.

In each cotyledon there is a median strand of provascular tissue flanked on either side by one or more smaller lateral strands (fig. 4). The number of laterals in the cotyledons varies, one or two being the usual number, although some cotyledons have been observed with one lateral strand on one side of the midvein and two on the other. In cross section the provascular strand of the midvein appears lobed, while the lateral strands are circular. The cells of the mesophyll region of the cotyledon are filled with large inulin sphaerocrystals, which take a deep stain, while the cells of the provascular strands are not so large and are not filled with these large crystals but contain a minutely granular substance which does not stain deeply.

At this age the phloem and xylem regions are not differentiated, but a little later in the ontogeny primary phloem ducts are differentiated, and concurrently with the formation of these ducts the first annular protoxylem elements are matured. The initial differentiation of vascular tissue occurs near the base of the cotyledonary petiole and proceeds both upward and downward. This development differs from that of the root in that the protoxylem of the root does not differentiate until after the primary phloem is well defined.

In tracing the provascular strands downward from the cotyledons through the hypocotyl to the meristematic region of the root, they remain separate to a level just below the point of divergence of the cotyledons. At this level the lateral strands occupy a more central position (fig. 5), and still lower in the hypocotyledonary axis the lateral strands from opposite cotyledons converge (fig. 6). This results in the formation of two strands in the cotyledonary plane on opposite sides of the axis of the hypocotyl (fig. 7). When there are two lateral strands on each side of the midvein, the outermost strands of each cotyledon converge to form a common strand, with their counterpart in the opposed cotyledon, and these in turn anastomose with the common strand of the inner laterals of each cotyledon (fig. 22).

The midvein strands, which appear lobed in cross section in the cotyledons (figs. 4-6), become ovate in outline near the base of the cotyledons (fig. 7). The two median strands then converge in the hypocotyl with the united lateral strands to form the fluted central



FIGS. 4-9.—Cross sections through young embryo. Provascular strands shown in black. *cls*, united lateral strand of opposite cotyledons; *ls*, lateral strand; *mvs*, midvein strand; *pvc*, provascular cylinder.

provascular cylinder (fig. 8). The lobed condition of this cylinder is less evident at lower levels (fig. 9) and the stele becomes circular in cross section. This cylinder of cells has at its center larger cells (fig. 2) whose cytoplasmic contents are less dense than those surrounding them.

#### VASCULAR ANATOMY OF HYPOCOTYL AND COTYLEDONS

The course of the vascular bundles of the cotyledons and hypocotyl is shown graphically (fig. 22). In the following description the bundles are traced from their point of initiation in the cotyledons to the diarch root. To avoid confusion, the median bundle is first discussed and then the course of the lateral bundles of the cotyledons is considered.

About midway between the base and the tip of the cotyledon of a three- to ten-day old seedling, there have been differentiated two vascular bundles which constitute the midvein. The two protoxylem strands of each bundle lie in close association with each other and the metaxylem is tangentially oriented with respect to the protoxylem (fig. 16). The phloem of each bundle lies in an abaxial position in relation to each metaxylem group. These two closely associated bundles are surrounded by a sclerenchymatous sheath of cells whose walls are distinctly thicker than those of the mesophyll cells.

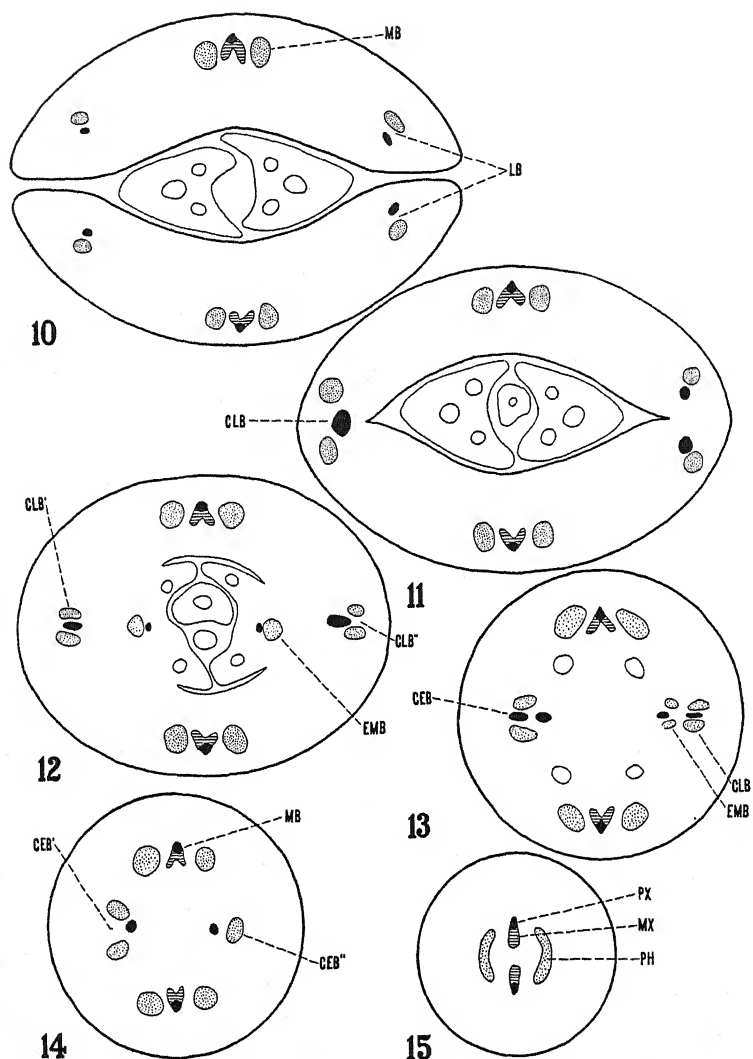
THOMAS (16, 17) discusses the double bundle, which sometimes constitutes the midvein of the cotyledons, in relation to types of transition found in certain seedlings. The double bundle is reported as consisting of two groups of primary phloem with a single strand of protoxylem between, the three groups being in radial arrangement; but no mention is made of the metaxylem of this bundle. In *Cynara scolymus* the metaxylem is very evident and the midvein is truly a double bundle located along the median line of the cotyledon and consisting of two separate strands, each of which contains protoxylem, metaxylem, and phloem. The vascular elements of these two distinct bundles, however, are inclosed in a common sheath (fig. 16). The two protoxylem strands unite near the base of the cotyledon, in the petiole, so that the bundle contains two groups of phloem, two groups of metaxylem, but only one common protoxylem group. At a lower level, at the base of the hypocotyl, the metaxylem

groups also unite, but the two phloem strands of each double bundle never unite to form a common strand. Instead one phloem strand on each side of the median bundle of one cotyledon unites with the equivalent phloem strand of the opposed cotyledonary median bundle to form the lateral phloem strands of the diarch root. The interpretation of the median bundle as a double one depends, therefore, upon the level at which sections are taken.

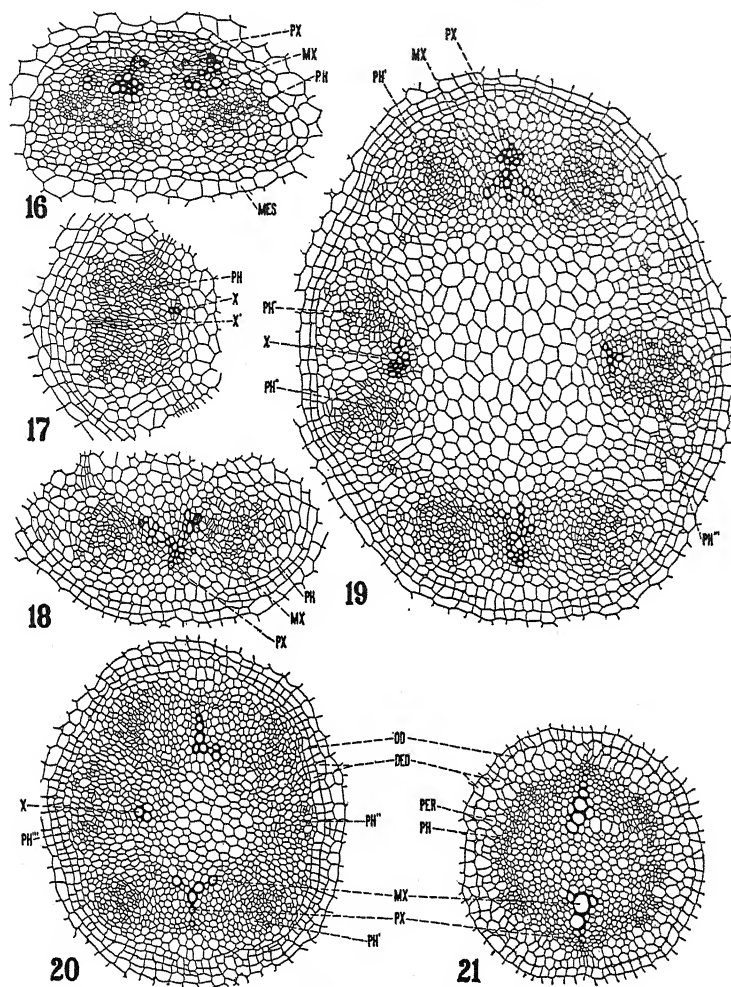
At the base of each cotyledon and below the point where the protoxylem strands of the double bundle have converged, there is formed a bundle which has the primary xylem in a V-shaped arrangement. The protoxylem lies in an abaxial position at the apex of the V and the two metaxylem groups form the arms. The strands of primary phloem lie on either side of the metaxylem (fig. 10mb). This arrangement of the median bundle is also found in the upper part of the hypocotyl (fig. 18). At successively lower levels (figs. 19, 20) in the hypocotyl the differentiation of metaxylem with reference to the protoxylem is more and more centripetal, which brings the two arms of metaxylem into closer association. The two strands of phloem still lie in a lateral position with reference to the primary xylem (fig. 14mb). At the base of the hypocotyl the primary xylem differentiates in the exarch manner of the root (fig. 21), and the two groups of phloem on each side of the intercotyledonary plane converge, forming a single strand of phloem on either side of the primary xylem (figs. 15, 21).

The lateral bundles of the cotyledons are endarch collateral (fig. 10lb), but at a level just below the divergence of the cotyledons they converge toward the cotyledonary plane, and the xylem of the lateral bundles of opposite cotyledons unites (fig. 11clb). This fusion bundle then consists of xylem flanked on either side, parallel to the intercotyledonary plane, by phloem (fig. 12clb'). The phloem becomes reoriented at a lower level (figs. 12clb'', 14ceb') and finally lies outside of the xylem and on the same radius (fig. 14ceb''). Ultimately this vascular unit ends blindly in the lower hypocotyl (fig. 20).

LEE (7) and SILER (12) both describe transitions very similar to that described here for *Cynara scolymus*. LEE discusses the transition of *Arctium majus*, while SILER discusses a like transition for *A. minus*. The main facts of the transition in these two species con-



FIGS. 10-15.—Diagrammatic representation of vascular anatomy of three- to ten-day old seedling. Primary xylem shown by black; metaxylem, lined; primary phloem, stippled. *ceb*, bundle composed of midvein of epicotyledonary leaf and combined lateral veins of opposed cotyledons; *clb*, combined lateral bundle of opposite cotyledons; *emb*, midvein of epicotyledonary leaf; *lb*, lateral bundles of cotyledons; *mb*, median bundle; *mx*, metaxylem of root; *ph*, primary phloem; *px*, protoxylem of root.



FIGS. 16-21.—Cellular details of transition and root: Fig. 16, structure of double bundle of midvein of cotyledon. *ph*, phloem; *px*, protoxylem; *mes*, mesophyll; *mx*, metaxylem. Fig. 17, epicotyledonary bundle converging with lateral cotyledonary bundle. *ph*, phloem of both bundles united; *x*, xylem of epicotyledonary leaf; *x'*, of lateral cotyledonary bundle. Fig. 18, cotyledonary midvein bundle in upper part of hypocotyl. Fig. 19, middle of hypocotyl. *ph'*, phloem of bundle in cotyledonary plane; *ph''*, in intercotyledonary plane; *x*, xylem of intercotyledonary bundle; *ph'''*, phloem of intercotyledonary bundles united. Figs. 20, 21, section at base of hypocotyl and root respectively. *ded*, double endodermis; *od*, oil ducts; *per*, pericycle; *ph*, phloem of root; *ph'*, of bundle in cotyledonary plane; *ph''*, in intercotyledonary plane (xylem has not differentiated); *ph'''*, phloem of opposite bundle; *x*, xylem of bundle in intercotyledonary plane.

form to those found in *C. scolymus*. The merging of the lateral cotyledonary bundles with the metaxylem of the cotyledonary midvein bundle which they describe for these species is not encountered in *C. scolymus*. Instead in the young seedlings these lateral bundles end

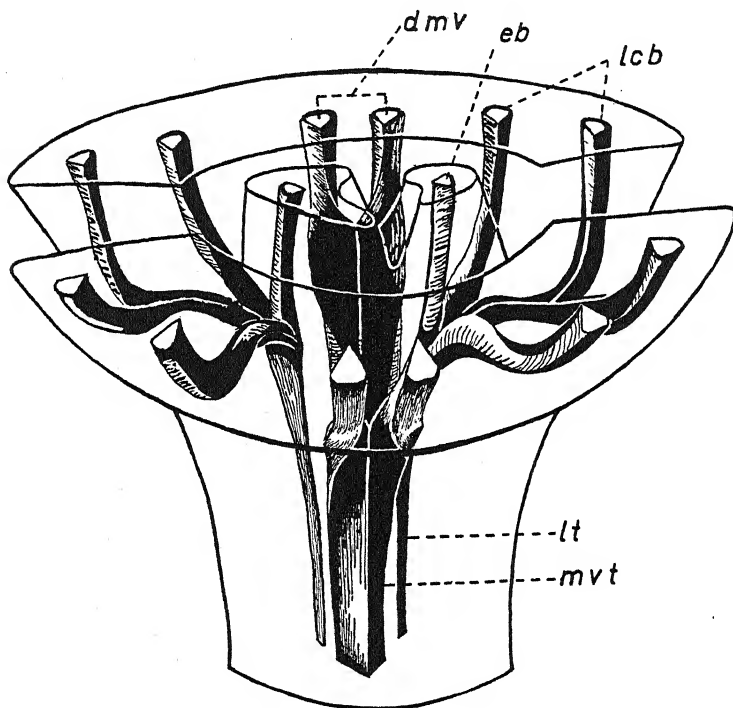


FIG. 22.—Schematic diagram of seedling vascular system showing two laterals on each side of midvein bundle. *eb*, epicotyledonary midvein bundle; *lcb*, lateral cotyledonary bundle; *lt*, lateral bundle of intercotyledonary plane; *mvt*, bundle of cotyledonary plane; *dmv*, double midvein bundle.

blindly in the hypocotyl. The only connection between these lateral bundles and the primary vascular elements of the root is by means of secondary tissues which are laid down by the cambium later in the ontogeny of the axis.

#### VASCULAR SYSTEM OF EPICOTYL

In a three- to ten-day old seedling the first two foliar leaves have developed provascular tissue consisting of a median bundle with a

lateral bundle on either side. At about the level of divergence of the cotyledons, the median bundle of the epicotyledonary leaf has differentiated as a collateral endarch bundle consisting of protoxylem and phloem (fig. 12emb). At a slightly lower level the phloem differentiates as two strands, which come to lie on either side of the xylem in a plane parallel to the cotyledonary plane (fig. 13emb). This midvein trace extends downward, and at about the middle of the hypocotyl unites with the cotyledonary trace, which lies in the intercotyledonary plane. The phloem strands of the combining bundles unite at a higher level (figs. 13ceb, 17) than do the xylem strands. After the xylem of the two bundles unites, the combined bundle consists of xylem flanked on either side by phloem. Near the base of the hypocotyl this bundle forms a collateral bundle by the convergence of the phloem strands (fig. 14ceb', ceb'').

### Summary

1. Germination of the seed and development of the seedling of *Cynara scolymus* are discussed.
2. The embryo shows well defined provascular strands which later differentiate vascular tissue.
3. The primary root is diarch with three clearly defined histogens. The plerome and periblem give rise to the stele and cortex respectively, while the root cap and epidermis are derived from a common histogen, the calyptogen-dermatogen.
4. The order of differentiation of the primary vascular elements is described.
5. The primary endodermal oil ducts arise schizogenously between the layers of the double endodermis.
6. In the transition, the primary xylem of the root in the cotyledonary plane is continuous with the xylem of the midveins of the cotyledons. The lateral bundles of the cotyledons end blindly in the intercotyledonary plane of the hypocotyl.
7. The structure of the double bundle of the cotyledon is described.
8. The midveins of the first foliar leaves anastomose with the lateral bundles of the cotyledons in the hypocotyl.



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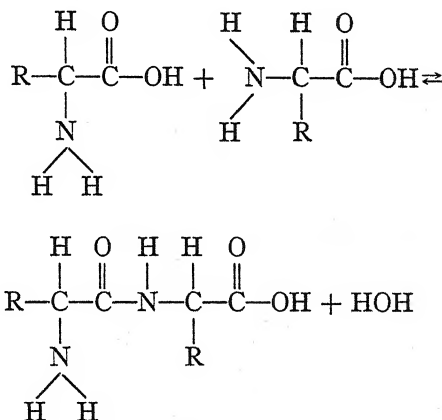
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# POTASSIUM AND CALCIUM IN RELATION TO NITROGEN METABOLISM<sup>1</sup>

G. T. NIGHTINGALE

In both plant and animal metabolism, the "protein sparing" action of carbohydrates has long been known and in plants has been repeatedly demonstrated by the experiments of SCHULZE and PRIANISCHNIKOV (15). As long as carbohydrate reserves are high there tends to be immobilization of organic nitrogenous reserves. With increase in content of dry matter there is loss of water from amino acids and condensation to polypeptides and insoluble proteins, thus:



Conversely, with decrease in carbohydrates, proteins are hydrolyzed to relatively simple, water soluble, mobile forms of organic nitrogen, which under favorable conditions are readily available for the synthesis of the proteins of meristematic tissue: proteins very different in quality from the storage proteins which are apparently the condensation products of amino acids. The cyclic structure hypothesis

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may perhaps lead to a better understanding of the nature of the proteins of meristematic structures, for it indicates that amino acids can enter into combinations that are not included in the conventional peptide hypothesis, which, as already pointed out, is primarily that of synthesis of proteins through chemical dehydration of amino acids.

In apparent contrast to the immobilization of organic nitrogenous reserves under conditions of carbohydrate accumulation, there is the work of MASON and MASKELL (9) on translocation of nitrogen in nitrogen deficient plants, that of MURNEEK (11) on autumnal migration of nitrogen from apple leaves, and that of RICHARDS and TEMPLEMAN (18) on transport of nitrogen from the lower leaves of nitrogen deficient barley plants. RICHARDS and TEMPLEMAN take exception to the general ideas expressed, on the basis that, under conditions of shortage of nitrogen, the plants should not be expected to store the limiting element. This is a convenient explanation but means little. Certain it is that if nitrogen deficient, high carbohydrate plants are shaded, shifted to darkness, or heavily pruned, there is, accompanying the decrease in carbohydrates, very rapid hydrolysis of proteins and re-utilization of the resulting amino acids to form new proteins of the meristematic regions. This is accompanied by a striking increase in volume of the plant even though there is no external supply of nitrogen available.

As MOTHES (10) shows, however, the several results mentioned are not necessarily in conflict. His extensive studies of the nitrogen metabolism of leaves fully corroborated the earlier work of SCHULZE and PRIANISCHNIKOV on the "protein sparing" action of carbohydrates (15). In addition, MOTHES found that old leaves (as the primary leaves of bean, even before they showed any external signs of senescence) exhibited proteolysis both on increase of carbohydrates in the light and on addition of glucose in the dark. Regardless of carbohydrate content, undoubtedly there is cleavage of protein as tissues approach senescence, and this often occurs in the more advanced stages of nitrogen deficiency and in maturing leaves. As a general thing, however, carbohydrate accumulation in plants is characterized by condensation and immobilization of organic nitrogenous reserves, and decrease in carbohydrates is correlated with

hydrolysis of stored proteins. Under favorable conditions the development of new tissues accompanies this hydrolysis of stored proteins.

The preceding remarks indicate in a general way the course of metabolism of organic nitrogenous materials already in storage in the plant. There are various external sources of nitrogen which plants can utilize, but under usual commercial field conditions the principal external source of nitrogen is undoubtedly nitrate.

In many plants nitrate may be stored in enormous quantities. Not infrequently nitrate in plant tissues may accumulate until a concentration is reached that is equal to or even greater than that of the organic nitrogen. However, the greatest storage of nitrate usually occurs, not under conditions most favorable for active protein synthesis, but under conditions which are less favorable, assuming of course an abundant external supply of nitrate.

Although all cell constituents necessarily exert an effect upon the plant, the presence of nitrate in itself will not result in the formation of new cells and growth. There must first occur reduction of nitrate to nitrite, then to ammonium, and eventually organic nitrogen synthesis. This involves an endothermic reaction with oxidation of carbohydrates or their derivatives and a greatly increased rate of respiration, as recently demonstrated by HAMNER (6). Clearly, new synthesis of organic nitrogen, if carried to excess, will tend greatly to deplete the carbohydrate reserves of the plant, especially under conditions of limited opportunity for carbon dioxide assimilation.

In connection with student instruction and in formal research experiments (12, 13, 14), the writer has had the opportunity, over a period of about ten years, repeatedly to observe effects of potassium and calcium deficiency on the growth and nitrogen metabolism of several different species of dicotyledonous plants.

POTASSIUM DEFICIENCY.—When plants are abundantly supplied with nitrate under conditions reasonably favorable for carbon dioxide assimilation, one of the first effects of lack of potassium is invariably that of nitrate and carbohydrate accumulation. Accompanying the increase in carbohydrates there is immobilization of amino acids. Externally the plants exhibit all the general symptoms usually included by the term nitrogen deficiency. "Leaf scorch,"

frequently exhibited by fruit trees (19), and various other symptoms sometimes considered to be more or less characteristic of lack of potassium, may be induced or very largely eliminated by varying the supply of other nutrient ions or the opportunity for carbon dioxide assimilation. During early stages the situation is also anatomically much the same as that of plants lacking an external nitrogen supply. Mechanical elements are strongly developed, but actively dividing cells are few and limited to apical tissues, which are the only regions where potassium can be found in quantity in plants lacking an external supply of this element. The cambium of tomato stems, for example, and the supernumerary cambiums of the storage roots of beet, sweet potato, etc., are essentially dormant. What growth occurs is therefore mainly in length and not in diameter, potassium being almost completely water soluble and freely translocated to the growing tips. A comparable response is exhibited by pineapple plants; except that, in this species, potassium when limited migrates to the relatively meristematic leaf bases and some other regions where active cell division occurs.

Carbohydrate and nitrate accumulation during the early stages of lack of potassium is associated with inability on the part of the plant to synthesize organic nitrogen from stored nitrate. This is indicated by very limited nitrate reductase activity, as determined by ECKERSON (3), and also by the fact that, when a supply of potassium is made available to potassium deficient plants, nitrite and amino acids appear and there occurs very rapid utilization of and decrease in nitrate as well as carbohydrates.

It is apparent that moderate limitation of potassium results in much the same symptoms and conditions as does a low level of nitrogen nutrition. Horticulturists and others have learned through empirical fertilizer tests to avoid excessive vegetative growth by limiting the supply of potash fertilizers. Clearly, if economically feasible, plants should be supplied with abundant potassium. Luxury consumption of potassium will not of itself produce excessive vegetative growth and depress the yield of fruit, as it is sometimes said to do. The desired quality of vegetative and reproductive growth can be much more efficiently attained by regulating the available nitrate supply.

There is considerable literature dealing with the effect of potassium on the structure of stems. Much of this is concerned with work done on grains to determine the influence of potassium upon stiffness of straw. The anatomical structure is recorded in most cases, but it is taken up entirely on the basis of the potassium treatment received by the plant, and any possible effect on the carbohydrate content is seldom considered. As might be anticipated, stiff straw is most frequently obtained, not necessarily when potassium is especially abundant, but under conditions of rather low nitrogen nutrition and carbohydrate accumulation in the plant. Potassium is frequently recorded as favoring the development of thick cell walls and stiff straw, but in many cases this element is reported as having the opposite effect. The reason is principally in the effect of a given treatment upon the rate and type of utilization of carbohydrate reserves (8, 20). Various workers have indicated that potassium is directly or indirectly essential for carbohydrate synthesis and hence for cell wall formation; but if nitrate is present in abundance and conditions permit rapid protein synthesis, the carbohydrate content of the plants will be low and cell walls thin regardless of the potassium present. In order to obtain cell walls of sufficient thickness and at the same time fruitful plants, the practical indication is therefore to apply enough potassium but to avoid an excess of nitrogenous fertilizers.

Under relatively extreme conditions of potassium deficiency, or following the initial stage of carbohydrate accumulation, in the practically complete absence of an external potassium supply there invariably occurs a marked decrease in content of sugars and starch. There does not seem to be failure to utilize stored carbohydrates, for HARTT'S (7) results and those of the writer indicate that digestion of carbohydrate reserves and translocation of sugars proceed unhindered in low potassium plants. On the basis of the work of GREGORY and RICHARDS (5), carbohydrate depletion would seem to be correlated with an unfavorable balance between assimilation and respiration of carbon dioxide.

The later stages of potassium deficiency are therefore usually characterized by carbohydrate depletion, and with rather rapid decrease in carbohydrates there occurs a typical proteolytic response

(not in the least peculiar to potassium deficiency) yielding amino acids and related compounds. These internal changes are made manifest externally by an increase in green color of younger leaves and the development of new stem tissue that is spindling, soft, and succulent, most of the potassium in the plant migrating to these newly developed structures. Shortly following these responses the plants often die, senescence being greatly accelerated if fruits are present, since the little available potassium in the growing vegetative tissues tends eventually to be translocated to the reproductive structures.

RICHARDS and TEMPLEMAN (18) did not obtain the carbohydrate accumulation stage in potassium deficient barley plants, although they cite the results of many workers who have obtained this response with various species. They conclude, as do PHILLIPS *et al.* (17), that there is no proof that potassium bears any direct relationship to the synthesis of protein in the plant. They suggest that the accumulation of amino acids in potassium deficient plants is possibly the reason for limited nitrate reductase activity. This may well be a dominant factor under many experimental conditions, especially if opportunity for carbon dioxide assimilation is limited by the prevailing light conditions or nullified by respiration of carbon dioxide during long nights at high temperature. At least in tomato, nitrate reductase is often very low prior to, as well as following, the proteolytic formation of amino acids. Often in low potassium plants in the later stages of deficiency the percentage of amino acids and related compounds is as high or higher than in comparable plants receiving abundant potassium. But the absolute amount of organic nitrogen is of course very low, as potassium deficient plants are relatively small.

Obviously the function of potassium is little understood and any effects recorded may well be indirect. Nevertheless in practical plant nutrition it is important to avoid a deficiency of either organic nitrogen or carbohydrates, and potassium directly or indirectly plays an important part in determining the amount and concentration of these materials found in the plant.

CALCIUM DEFICIENCY.—Although the respective initial symptoms of lack of nitrogen, magnesium, phosphorus, and potassium do not

usually appear simultaneously in a given series of plants, the effect upon their external appearance, especially during early stages, is often very similar but may vary materially with the character of the nutrient or soil solution and opportunity for carbon dioxide assimilation. In tomato and in apple (1, 13, 14) grown in sand culture, in all four cases of nutrient deficiency the older leaves were typically yellowish green and the younger leaves and tip of the stem were fairly dark green and usually remained so for a considerable period.

On the other hand, the appearance of calcium deficient tomato plants and apple trees was very distinctive, in that the upper part of the growing tips of the tomato plant and the distal portion of the current twig and leaf growth of apple trees was yellowish, as a rule, whereas the first formed leaves remained relatively dark green even after the stem tip was dead or all elongation had ceased. These results are not peculiar to the species mentioned, for almost identical responses have been recorded for other kinds of plants (4). Boron deficiency is also associated with injury to young rather than to mature tissues (2).

The root systems of calcium deficient plants were drastically affected. Frequently the roots practically failed to develop and those that did appear were characteristically short, bulbous, and brown at the tips, with sloughing off of cells farther back. The roots were short because of a slow growth of the meristem, and bulbous because cortical cells enlarged somewhat more laterally than longitudinally. The failure of lateral root primordia to develop sufficiently to emerge through the cortex also contributed to the bulbous appearance.

When new cells are formed, there must not only be calcium presumably for development of the middle lamella, but also available calcium for combination with materials of the protoplast. When calcium was deficient, granular proteinaceous inclusions accumulated and there was only limited absorption and reduction of nitrate.

Plants possess in a considerable degree the capacity to re-utilize contained nitrogen, magnesium, phosphorus, and potassium. Each of these elements when limited tends to migrate from mature to relatively meristematic zones of roots and tops. However, as PETRIE'S (16) work indicates, calcium is much less mobile in the plant than



the elements mentioned; it accumulates in the leaves but it does not freely migrate from them again, so that the supplies for each successively formed structure must be derived very largely from the nutrient or soil solution.

This accumulation of immobile calcium occurred in tomato and apple not only in the comparatively dark green lower leaves but in the older portions of stem tissue and roots. Calcium was stored in the form of calcium oxalate and also in combination with proteins or other materials, as "combined calcium." Although tomato plants which received an external supply of this element contained considerable amounts of mobile, water soluble calcium, nearly 100 per cent of the calcium of the fresh tissue of the calcium deficient plants was water insoluble and most of it was located in the older tissues of roots and tops. Nevertheless the utilization of calcium oxalate and the re-utilization of combined calcium took place so slowly that root and stem tips died while there were yet heavy deposits of calcium oxalate and a high concentration of combined calcium in the older tissues.

The nature of combined calcium has not been determined, but it should be emphasized that it is released and made available to meristematic tissues, and new growth occurs following any treatment, such as continuous darkness, shading, or pruning. Calcium deficient plants are often very high in carbohydrate reserves, but carbohydrates can readily be decreased on shifting such plants to darkness or by defoliation or pruning. Accompanying digestion and decrease in stored carbohydrates there is a typical proteolytic response, release of combined calcium and translocation of the resulting soluble calcium to the developing organs, along with the hydrolytic products of protein and carbohydrate reserves.

This response in calcium deficient plants on decrease in carbohydrates and likewise on shift from minus to plus calcium nutrient treatment is apparently similar in principle and effect in certain respects. In both cases soluble mobile calcium is made available for combination with newly formed proteins or other materials. A supply of amino acids for formation of new proteins of meristems is likewise available in both instances in the low carbohydrate,

calcium deficient plants through proteolysis and in the plants newly supplied with calcium through nitrate absorption and assimilation.

Certain responses associated with calcium deficiency have been recorded, but it must be admitted that little is known concerning its function. Lime is commonly used to neutralize excessive soil acidity, but non-acid soils are sometimes deficient in calcium. Often during a period of drought the calcium content of certain soil solutions apparently becomes very low. At least the roots of fruit trees in the field exhibited all the external symptoms of a deficiency of this element and contained little or no uncombined calcium. Especially in the case of apple and peach trees, a deficiency of calcium may be very drastic, owing to its effect on the fine rootlets, which are the organs chiefly concerned in the initial phases of protein synthesis in the fruit trees mentioned (14, 15).

PINEAPPLE EXPERIMENT STATION  
HONOLULU, HAWAII

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# HISTOLOGICAL REACTIONS OF BEAN PLANTS TO GROWTH PROMOTING SUBSTANCES

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(WITH FIFTY-THREE FIGURES)

## Introduction

In previous papers (2, 5) some of the histological responses of the bean and of the tomato to applications of indoleacetic acid in lanolin were described. Studies on the bean, which responds readily to wounding (6) and environmental changes, and other plants have been continued and further details with reference to the former are presented herewith. In general the same techniques have been followed, except that in addition to terminal application of lanolin mixture (30 mg. indoleacetic acid per gram of lanolin) to decapitated stems, the same mixture has been applied as a narrow band completely encircling the uninjured internodes of stems, to the slightly abraded surfaces of young pods along the adaxial suture, and over the cut terminal surfaces of similar pods. Also two additional substances, indolebutyric acid and naphthaleneacetic acid, both received through the courtesy of Dr. P. W. ZIMMERMAN of the Boyce Thompson Institute, have been used in mixture with lanolin.

## Investigation

### OLD TERMINAL TUMORS RESULTING FROM APPLICATION OF 3 PER CENT INDOLEACETIC LANOLIN MIXTURE

A report has been made (5) upon the developmental stages of apical tumors resulting from application of a 3 per cent mixture of indoleacetic acid in lanolin up to a period of 168 hours following application (fig. 1). Such tumors may continue to grow and develop for periods longer than six months, frequently attaining a diameter

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of 2 cm. and more and a total height above the original cut surface of the stem of 2–2.5 cm. (figs. 2, 3). The surface of such old tumors is tuberculated or warty, each tubercle or wart generally tipped with

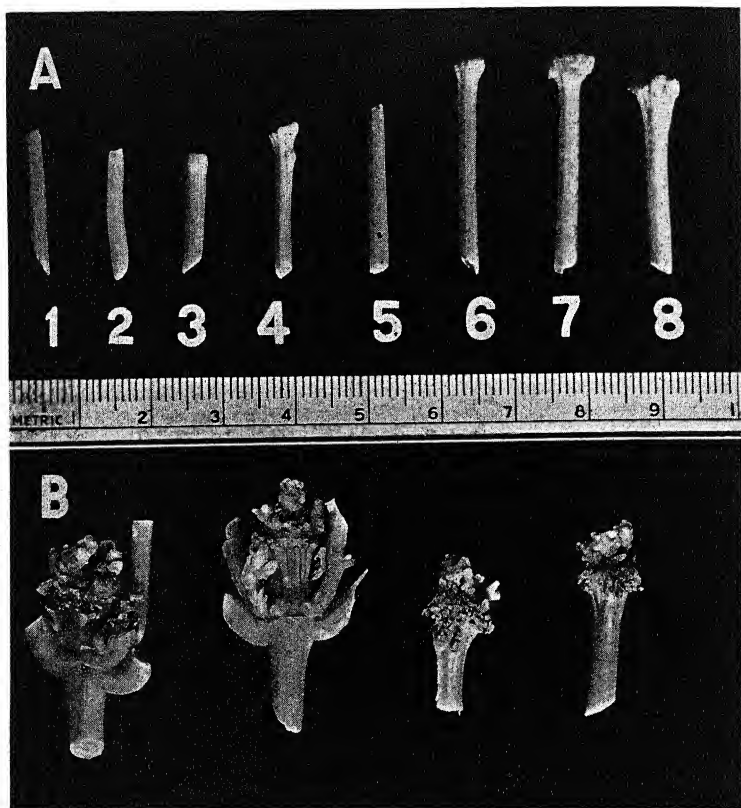


FIG. 1.—Stages in tumor development (time given in hours after treatment with 3% indoleacetic acid lanolin mixture). A: 1, just after decapitation; 2, 30 hours; 3, 48 hours; 4, 66 hours; 5, 72 hours not treated; 6, 72 hours treated; 7, 144 hours; 8, 168 hours. B: four apical tumors 28 days after decapitation and treatment of stem. Adventitious roots have not emerged, but prominent ridges and rows of small tumors over vascular bundles are evident, as is also central apical mass of proliferating tissue. Natural size.

a bit of lanolin mixture which apparently slowly supplies the indoleacetic acid which acts as a stimulus for continued development. As development proceeds the crown of roots and root primordia be-

comes more or less completely imbedded by the expanding tissues. If the surrounding atmosphere remains relatively dry, little development is made by these roots and frequently many of them die or partially disintegrate; but if the surrounding atmosphere is saturated with moisture, or if wet sand or other moist medium is placed in contact with the zone of roots, they develop actively, grow out for an indefinite length from the stem or tumor, and produce secondary roots in abundance.

Microscopic inspection of the old tumor above the root zone shows that it was developed largely from proliferated tissue of the pith (figs. 3, 4), although parenchymatous cells of the phloem and xylem which also remained meristematic gave rise to derivatives which contributed to its bulk. As the tumor progressively enlarges and the lanolin mixture becomes somewhat dry and toughened, this covering mixture becomes fragmented into irregular bits or small masses. Directly beneath such a mass the cells proliferate, expand, and differentiate more rapidly, with the result that the surface becomes more and more tuberculated and the separated small masses spread farther apart. There is, however, decided activity on the part of many of the cells at the base of and between the tubercles, so that a considerable volume of tumor is also built up between them. Some derived cells differentiate into strands of xylem, phloem, or both, with zones of active cells between and around them, so that the whole structure becomes highly vascular, anastomosing strands and patches of xylem, phloem, and parenchymatous cells occurring in every conceivable relation to one another. Many of the parenchymatous cells remain active and more or less continuously give rise to derivatives, some of which in turn differentiate into tracheids, sieve tubes, companion cells, or other vascular elements. At the periphery of a tumor most of the cells become suberized, but directly underneath these others divide actively and from them vascular elements are differentiated. In observing a cross section through such a developing tumor, one is strongly reminded of a similar section through a fleshy root of a sweet potato or a radish the secondary xylem parenchyma of which has undergone or is undergoing tertiary thickening.

As previously stated, some of the adventitious roots may die;

others may become much compressed or imbedded owing to the activity of the phloem cells and phloem derivatives (figs. 4, 5). Slightly below the root zone, the stem may enlarge but little, and secondary thickening proceed in the usual manner. Centrifugal to the secondary phloem of many of the principal vascular bundles vascular strands originally derived from endodermal or primary phloem cells may continue to develop, thus approaching in general appearance the original stelar bundles. Such vascular strands frequently extend more than 10 cm. below the original treated surface (figs. 5, 6), although they rarely exceed 6 or 8 cm. in length. From the parenchymatous tissues which partially compose these outermost bundles root primordia may be initiated, and if ample moisture is provided, continue development for an indefinite period. In other respects the stems at 1-10 cm. below the apical tumor appear as do stems which have not been treated and have undergone the usual type of secondary thickening.

TUMORS RESULTING FROM LATERAL APPLICATION OF 3 PER  
CENT INDOLEACETIC ACID LANOLIN MIXTURE TO  
SECOND INTERNODE

The lateral applications to the second internode of the stem were made on plants the same age as those receiving the terminal applications at the time the first trifoliate leaf was beginning to spread out. The same strength of mixture was employed. By the use of a small pointed scalpel the mixture was drawn out into a thin thread, and this thread was then carefully laid on the stem so as completely to encircle it. Great care was used to avoid any possible mechanical injury to the surface of the stem, and through the method employed this was easily accomplished. In a few instances the line of application was marked with India ink, but this was needless, and in fact when the ink lay directly under the mixture, reaction of the stem at that point was frequently delayed.

Within 30 hours after application the internodes showed decided bending in one direction or another, but usually straightened out within 48 to 72 hours. The trifoliate leaf was bent downward at first but soon assumed its original erect or horizontal position. Marked yellowing and swelling of the stem occurred 2 or 3 mm.

above, below, and at the line of application. Such effects became more marked during several successive days, until by the end of a week or ten days the tumors were often 2 cm. or more long, spindle shaped, and markedly ridged over the vascular bundles. The line of mixture became stretched both vertically and in circumference to a band 2 to 3 mm. wide. Through this band and also above and below it, roots emerged, occurring in longitudinal rows of five to fifteen or more, mainly between the swellings over the vascular bundles. Under average greenhouse conditions these rarely emerged more than 2 to 3 mm. beyond the surface, but would grow apparently indefinitely long in saturated atmosphere. In nearly every case the swelling and the number of root primordia initiated both above and below the line of application were about equal (fig. 13). In no case did swelling or root differentiation occur as far below this line as was the case when the mixture was applied to the cut surface of decapitated stems. Very different results occur when the plants are grown continuously in the dark, but such results will be presented at another time.

If the atmosphere was not saturated with moisture the tumors apparently ceased active expansion in ten days to two weeks and became dull brown in color instead of light yellow; the surface cracked slightly. Almost without exception, in many different experimental lots and at different seasons, this quiescent stage extended over a period of about two weeks. Some of the tumors never grew further, although the cells composing them remained alive, but most of them again became active. The longitudinal cracks then became wider and the tumors grew rapidly. Winglike projections developed either from one side of the tumor or about its whole periphery. On their surfaces these wings carried out bits of the lanolin mixture. The rate of growth at various places became still more irregular, resulting in a rough tuberculated contour closely resembling that of apical tumors. At times growth was rapid, again slow; often a sort of rhythmic development took place. By the end of four to six months these lateral tumors measured 1-4 cm. in greatest diameter, and were still actively developing (fig. 9).

The presence of these tumors brought about little or no retardation of the growth of the stems, either axial elongation or expansion



in diameter, and the plants fruited as well and as abundantly as plants which had not been treated (figs. 7, 8).

The histological response of uninjured stems treated laterally closely resembled those of stems decapitated and treated terminally, although there are some striking differences. Within 30 hours after treatment, the cortical cells of laterally treated stems had elongated radially and by 48 hours the cells of the endodermis had begun division (fig. 11). Within 72 hours there was marked meristematic activity of some of the inner cortical parenchyma, the endodermal derivatives, the primary phloem, the cambium, and the cells of the rays flanking the phloem. The ray cells adjacent to the xylem and the pith enlarged somewhat but showed no pronounced meristematic activity at any time. This behavior of the pith is in sharp contrast to that of stems decapitated and treated apically. By 96 hours the root primordia were clearly defined in the outer portion of the rays. The phloem parenchyma proliferated markedly, as did also the cambial derivatives. Many of the outer cortical cells became stretched and torn; in some instances vascular strands were initiated from endodermal and phloem derivatives. The pericyclic cells showed some meristematic activity but little progress toward maturation as fibers.

By the ninth day (216 hours) the root primordia were well differentiated and may or may not have pushed through the surface; the derivatives of the cambium matured either as elements of the phloem or xylem; but there was little or no activity of the ray cells adjacent to the primary xylem, nor did the pith cells become meristematic. Centripetal to the bases of the roots and the inner portions of the primary phloem the whole stem appeared much like one of the same age which had received no treatment with mixture, except that the cambium was less well defined and the secondary xylem thinner walled (fig. 14A).

As previously stated, at about this time marked cell proliferation became greatly diminished and the whole stem showed relatively little change except for thickening of cell walls, some additions to the secondary xylem and phloem, and the number of phloem cells between the adventitious roots. On the twenty-third day it appeared much the same as on the ninth day (fig. 14B). In fact, further

marked changes did not occur for weeks or months in some plants, but in others the proliferated parenchymatous cells of the primary phloem and of the outer portions of the secondary phloem became more or less abruptly highly meristematic. Some of the derivatives matured as tracheids, others as vascular elements. Both xylem and phloem differentiated in every conceivable arrangement and condition of anastomosis, with highly active embryonic cells scattered among and exterior to these vascular strands and masses. Thus a mechanism becomes established whereby there may be continued cell proliferation, and from such derivatives vascular elements may continue to differentiate apparently indefinitely (fig. 15*A, B*). The adventitious roots became imbedded by these surrounding flanges and tubercles of proliferating tissue, although they were readily stimulated to further development if the tumors were surrounded by moist atmosphere or other moist medium. Under the latter conditions they pushed out through the surrounding masses of tissue and grew to indefinite length. Among the number of old tumors sectioned up to five months after treatment, the pith cells never have shown conspicuous activity. Rather they appeared much as they would in an untreated stem. Sometimes they contained a few starch grains near the xylem and were often dead, torn, and more or less disintegrated at the center of the stem (fig. 17).

#### APICAL TUMORS RESULTING FROM APPLICATION OF INDOLE-BUTYRIC ACID LANOLIN MIXTURE

For these experiments the plants were grown, decapitated, and treated just as were those plants used in the experiments on tumors resulting from indoleacetic lanolin mixture. The mixture was made up by adding 15 mg. crystalline indolebutyric acid to 1 gm. pure anhydrous lanolin at its melting point, stirring the two together thoroughly until a homogeneous mixture was obtained, and continuing such stirring and mixing until the whole mass solidified, thus avoiding the possibility of settling out of the indolebutyric acid.

In general the responses of the stems to this mixture are similar to those to 3 per cent indoleacetic acid lanolin mixture, with certain conspicuous exceptions. Within 48 hours the cortical cells showed marked enlargement, the endodermis and many cortical cells

adjacent to it proliferated, the primary phloem cells became active, and a few of the ray cells became meristematic near the cambium. By 72 hours the endodermal cells had proliferated very greatly, apparently much more actively in some areas than others, especially over the larger vascular bundles; the phloem was very active; in places the beginning of vascular strands from the phloem derivatives was evident. The rays were active, but there was only slight activity of the pith and that adjacent to the xylem vessels. By 112 hours the young roots were well defined, more frequently opposite a ray (fig. 20) but often over the phloem of one of the larger vascular strands (fig. 22). The pith cells had become highly meristematic and from them vascular strands and masses were differentiated; others continued actively meristematic and from their derivatives the process was repeated over and over. Thus through a period of time large apical tumors were developed, closely resembling those resulting from applications of indoleacetic acid lanolin mixtures.

A striking characteristic resulting from apical treatment of stems with indolebutyric acid is the broad heavy roots which are produced, and the fact that many of these develop over the phloem instead of from the rays; or often they may be very broad and involve not only the mass of cells in the phloem of a vascular bundle but also parts or all of the two adjacent rays. Such roots first begin in the endodermal cells over the phloem (fig. 21*B*) and gradually the cells of the phloem itself become involved. The xylem of such adventitious roots usually differentiates from derived cells of the rays adjacent to the phloem, and thus a connection with the xylem elements of the vascular bundle is effected. In many instances the phloem derivatives or endodermal derivatives themselves differentiate as vascular strands over the phloem of the vascular bundle, and the vascular system of the young root which lies centrifugal to them differentiates in such a manner that its conductive system is directly connected with a vascular strand or strands which lie centripetal to the vascular bundles of the main stem. Roots formed in this way frequently die at an early age, although under appropriate environmental conditions, they may continue to grow and develop as do those derived from the rays and endodermis (fig. 23).

The effects of the indolebutyric acid seem to be manifested at greater distances down from the surface of application than do those from indoleacetic acid, and very frequently the adventitious roots occur in three or four tiers close together near the cut surface, and a few scattered ones may appear in a circle 5 to 10 mm. below the surface of application (fig. 25).

APICAL TUMORS RESULTING FROM APPLICATION OF NAPHTHALENEACETIC ACID LANOLIN MIXTURE

The naphthaleneacetic acid lanolin mixture was prepared in the same manner as usual, except that only 5 mg. of the substance to 1 gm. of lanolin was used. The plants employed and methods of application did not differ from those in other experiments. The main gross and histological effects resulting from application of this mixture are similar to those of indoleacetic or indolebutyric acid. There are two obvious differences. First is the far lesser tendency to produce a vascular apical tumor above the surface of application, the tumors usually being relatively wide and flat topped with slightly scaly but not tuberculated surfaces. The second striking difference is the marked swelling of the tissues over the principal vascular bundles for a distance of 1 cm. or so below the treated surface, this swelling then terminating abruptly, so that a distinct ring of bumps or a conspicuous shoulder is formed. At each of these bumps one to several large root primordia are developed. Below these points swelling is much less but is still obvious to 8-10 cm. below the cut surface (fig. 30).

Histologically there are also some striking differences. The cortical cells, even those immediately below the epidermis, proliferate markedly. The endodermal cells are extremely active; the pericyclic cells proliferate but slightly, but the phloem, ray cells, and cambium derivatives become highly meristematic. The pith cells show much less response. By the end of 48 hours the pith cells next the protoxylem and metaxylem had dense content and showed a few divisions, and by the end of 72 hours there was a slight increase in the number of such derivatives, especially close to the surface of application, but 1-2 mm. from it there was little proliferation. By the end of 96 hours root primordia were evident in the rays; the phloem

and cambium derivatives had greatly increased, but generally there was no continued increase in activity of the cortical or endodermal cells. At the end of 120 hours after application, the young roots were clearly outlined, almost exclusively derived from the rays. The pith cells showed little activity except immediately adjacent to the primary xylem vessels, although at 215 hours the pith was markedly meristematic at a distance of one or two layers of cells beneath the surface of application. From such derivatives of the pith vascular masses were differentiated (fig. 29). Just below this zone the pith cell showed only occasional divisions.

As has been stated, the endodermal cells are generally extremely meristematic. From their derivatives over each vascular bundle large vascular strands differentiate and these are progressively enlarged from the cambium-like cells between their xylem and phloem. At the periphery of these strands the endodermal cells continue to proliferate actively and give rise to additional strands farther and farther away from the center of the stem. Some of these large masses of endodermal derivatives, however, lying a centimeter or so below the treated surface, together with the derivatives of two or more adjacent rays, may give rise to a single huge root primordium or to several primordia so close to one another that they appear more or less fasciated (fig. 30). These are the conspicuous bumps referred to in the gross description of these tumors.

As previously stated, activity of the endodermis and phloem parenchyma may extend for considerable distances down the stem, but the pith seems never to become as active as it does in tumors resulting from applications of indoleacetic acid or indolebutyric acid mixtures. Rather its cells mature, generally die at the center of the stem, and finally become disrupted and disintegrated.

#### COMPARISON OF TUMORS RESULTING FROM THE THREE GROWTH SUBSTANCES USING EQUIVALENT CONCENTRATIONS

As most of the experiments were carried out with indoleacetic acid (30 mg. per gram of lanolin), indolebutyric acid (15 mg. per gram), and naphthaleneacetic acid (5 mg. per gram), the question arose as to whether the characteristic differences shown by the tumors would

persist when all three substances were used at the same concentration. To test this, each substance was mixed with lanolin at the rate of 15 mg. per gram. All plants used were as nearly uniform as possible, and all applications were made at the same time. The results showed that the characteristic differences did persist (fig. 32). The higher percentage of naphthaleneacetic acid proved somewhat more toxic than the lower, with the result that the cells for several layers below the surface of application were killed (fig. 33), but beneath these the reactions were characteristic. Three weeks following application, the tumors resulting from application of the 1.5 per cent indoleacetic mixture were practically identical with those from the 3 per cent mixture; and those from the stronger concentration of naphthaleneacetic acid showed no more tendency toward proliferation of the cells of the pith and the building of a large apical vascular tumor than did those resulting from the use of the lower concentration (fig. 34).

#### TUMORS RESULTING FROM APPLICATION OF 3 PER CENT INDOLE-ACETIC ACID MIXTURE TO PODS

As shown previously (5), the pod of the bean responds actively to application of indoleacetic acid in lanolin. Bean pods have long been used as experimental material in testing growth substances (7, 4). We decided, therefore, to extend our histological studies to them. In all cases here reported a 3 per cent indoleacetic acid lanolin mixture was used. At first pods of various sizes and ages were used, and all responded; but it was finally decided to study those most critically which had attained a length of approximately 5 to 7 cm. and a suture to suture diameter of approximately 5 to 7 mm. (fig. 37B).

Two types of application were made. In one the pod was very lightly rubbed with a piece of fine emery cloth along the adaxial suture in order to remove the hairs from the surface and just slightly abrade the epidermal cells (fig. 44A). The mixture was then spread thinly and evenly along the suture, great care being taken to cause no further mechanical injury to the pods. Response was prompt and decisive (fig. 36). Pods not brushed with emery cloth respond to applications of the mixture also, but such response is far less uniform throughout their length unless the mixture is pressed down firmly

with the spatula to make sure that it comes evenly in contact with the epidermal cells. The other method of application consisted of cutting off the pod squarely with a sharp knife about one-fourth its length back from the tip, blotting off the exudate with lens paper, and then applying evenly over the cut surface a thin film of the mixture. Care is required in doing this to avoid crowding the mixture into the cavity of the pod, thus bringing about an uneven distribution over the exposed surface.

The gross responses of pods may differ appreciably in degree. In general larger and more extensive tumors are produced on pods during periods of bright rather than dull weather, and on the vigorous and vegetative plants rather than on those which are spindling or old and making little growth. Pods to which the mixture has been applied along the adaxial suture frequently develop no seeds, although they may do so if the mixture is applied after the seeds are about halfgrown. Those which had the mixture applied to the cut end produced seeds in at least half the cases in our experiments. In general if the seeds continue to develop the apical tumor is smaller than on those pods in which the seeds fail to continue development (fig. 35). Generally also, if a pair of pods is selected, one of them treated and the other not treated, the treated one will remain more or less succulent and green over a longer period than will the untreated. Several apically treated pods produced tumors and roots which, lying in contact with moist soil, continued to develop. These rooted pods remained green for nearly four months, finally dried and dehisced. The seeds fell from them, although the tumors at their tips remained green and continued to enlarge for more than two months longer, when they finally ceased to grow and decayed.

Figure 37 illustrates three stages in the development of the pod and seed of the Red Kidney bean. The transverse section shown at *A* is from a fruit shortly after fertilization has taken place. The petals have withered and the pod has begun enlargement. The adaxial suture is at the right, the abaxial at the left. For convenient reference the main body of the pod may be divided into an endocarp, indicated by the two or three innermost rows of blocklike cells, a mesocarp several cell layers thick and including the entire system of vascular bundles with parenchymatous cells between them, and

an exocarp several cell layers wide and without vascular strands. At *B* a similar pod several days older is shown. This is the stage of development of the pods when the mixture was applied to them. The three zones are even more clearly delimited, especially the endocarp which is several cell layers thick. The endocarp is apparently a much proliferated epidermis entirely without vascular elements and in the fresh pod is of a translucent or slightly water soaked appearance. *C* represents a transverse section through a nearly mature pod and seed. The endocarp has lost its succulent character and has shrunken against the mesocarp. The cells of the exocarp have also dried out and shrunken appreciably. Such a pod under average greenhouse conditions would become dry and dehisce within four to six days subsequently.

TERMINAL APPLICATION.—While there is some range in the time and degree of response of pods on different plants when grown under average greenhouse conditions, this is not great. Within 30 hours after application the parenchymatous cells of the exocarp and mesocarp adjacent to the treated surface have become yellowish, enlarged appreciably, and frequently a few divisions in various planes have taken place. By the end of 48 hours the number of such divisions has increased markedly, and a few of the cells of the endocarp may also show divisions, although these are uniformly slower in response than the cells of the other two regions. By the end of 96 hours (fig. 38) the parenchymatous cells of the exocarp and mesocarp have proliferated markedly and some of the derivatives of each region have begun to differentiate as vascular elements, so that the clear line of demarcation between the two regions is more or less obliterated. The cells of the mesocarpic portion are smaller than those of the exocarp. The cells of the endocarp near the surface of application also have begun to proliferate, but lag far behind those of the other two regions, with the result that the edges of the pod protrude markedly beyond the central portion. The endocarp remains completely non-vascular. Stages five and six days after application showed progressive development of the two outer regions, with marked increase in vascularity of the proliferated portions, and in many specimens a great increase in activity of the cells of the mesocarp also. By the end of twelve days the two outer portions



had so greatly proliferated that they not only flared markedly outward but also inward and over the endocarp, their edges close together. The whole apical tumor as seen externally consisted of proliferated cells derived from the exocarp and mesocarp (fig. 41A). During this same period primordia of roots, derived mainly from the derivatives of cells of the exocarp (fig. 41) but also from those of the mesocarp, differentiated. These generally protrude from the surface of the tumors. Such proliferation of the cells of all the regions may continue for several weeks, with increasing vascularity of the tissues derived from the exocarp and mesocarp; but none of the cultures showed any differentiation of vascular structures in the endocarp at any stage of its development. This is of some interest in that BONNER (1) has stated that similar material from the pod of the bean grown by him in tissue culture continued to grow and develop as parenchymatous tissue, and there was no differentiation of it into root and shoot nor into vascular tissue.

The cells of the exocarp and mesocarp do not respond to any great extent below the surface of application of the mixture. Apparently the indoleacetic acid is conducted but slightly through either the phloem or xylem. This effect is in contrast to the condition prevailing in the stem. That the cells near the surface of the endocarp show an apparent response at appreciable distances from the cut surface may result from the spreading of the mixture, or of some of the indoleacetic acid which may diffuse from it, over the surface of the endocarp which becomes moistened by the exudate, which is often copious at the time of cutting or for a considerable period thereafter. In fact, the response of the endocarpic cells to the treatment at some distance from the cut surface may be more apparent than real, for these cells show definite proliferation in pods that have been only cut and not treated, or in those left to develop with no treatment whatsoever.

APPLICATION ALONG ADAXIAL SUTURE.—After the mixture had been applied along the adaxial suture, the cells of the exocarp near the surface of application became meristematic, the divisions occurring at first mainly tangentially, then in all directions. By the end of the third day after application, some of the derivatives of these cells began to mature as vascular elements in scattered groups or

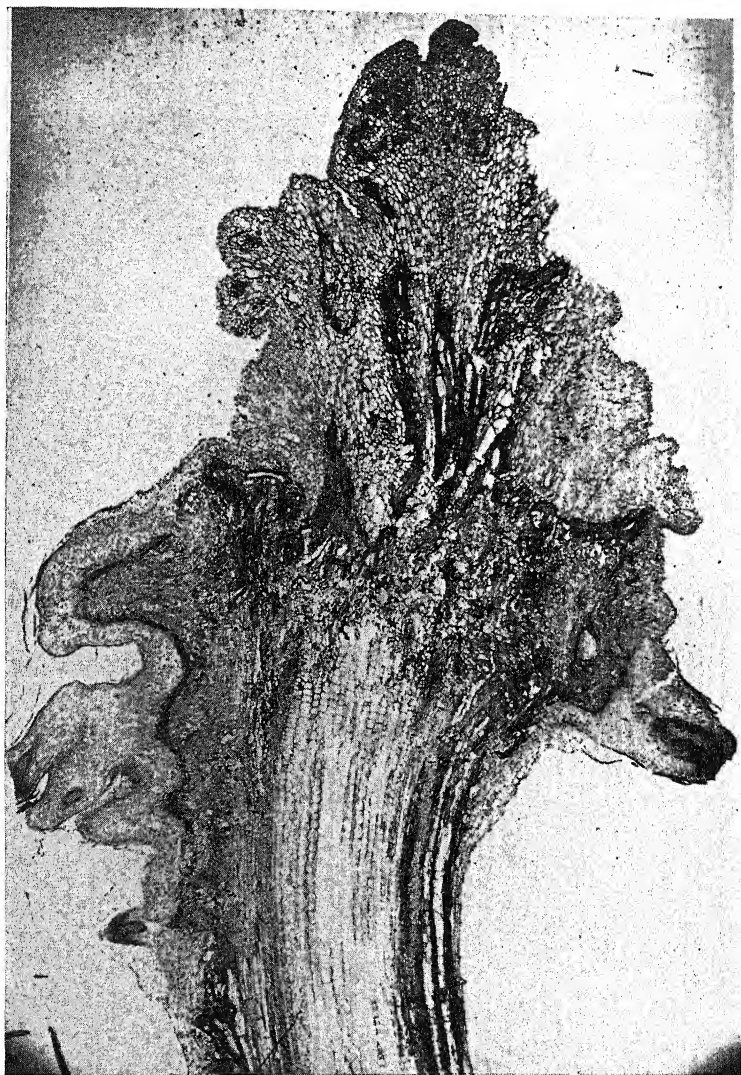


FIG. 2.—Apical tumor 31 days after treatment with 3% indoleacetic lanolin mixture. Nearly all vascular mass above level of application indicated by upper portion of zone of roots has been derived from the pith. Activity has continued to extend down stem also, with consequent differentiation of root primordia at considerably lower levels.

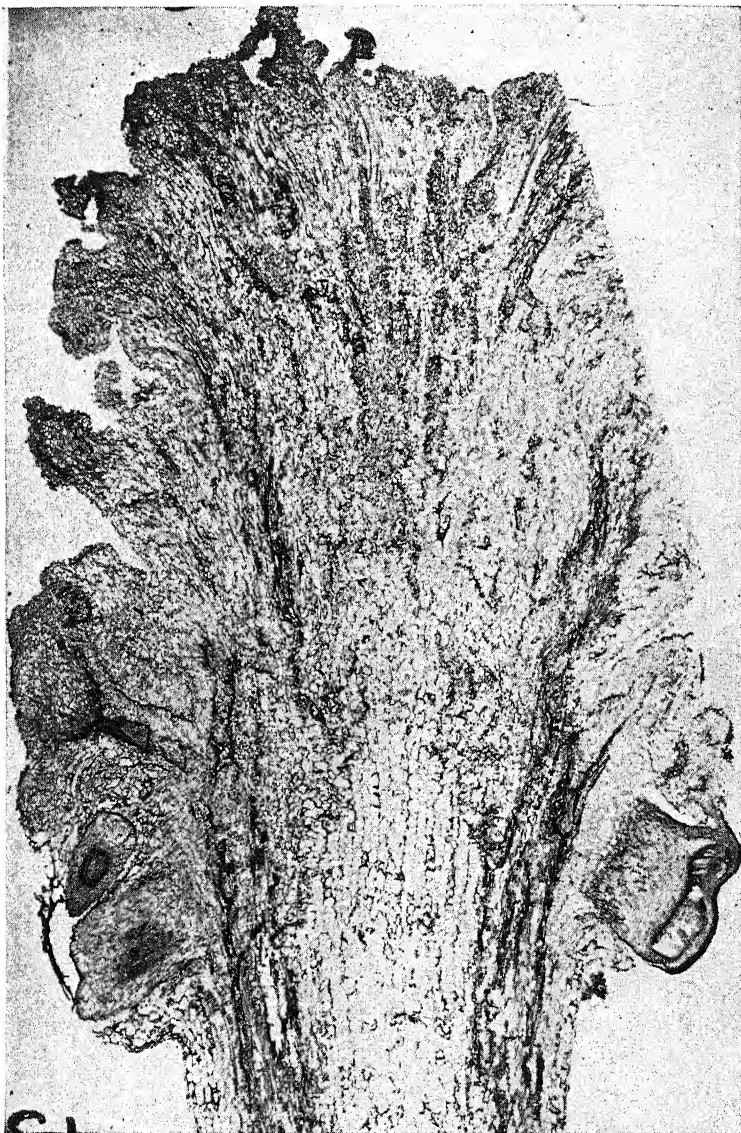


FIG. 3.—Same type of tumor 41 days after application of lanolin mixture. Most of vascular mass above surface of application is from the pith, that among and overtopping adventitious roots is mainly from the phloem. Pith shows very slightly increased meristematic activity or vascularity a short distance below original cut surface. Tubercles at top and periphery of tumor were still tipped with some of originally applied lanolin mixture and growing actively. Details of this activity were of same pattern as shown in figs. 50-53.

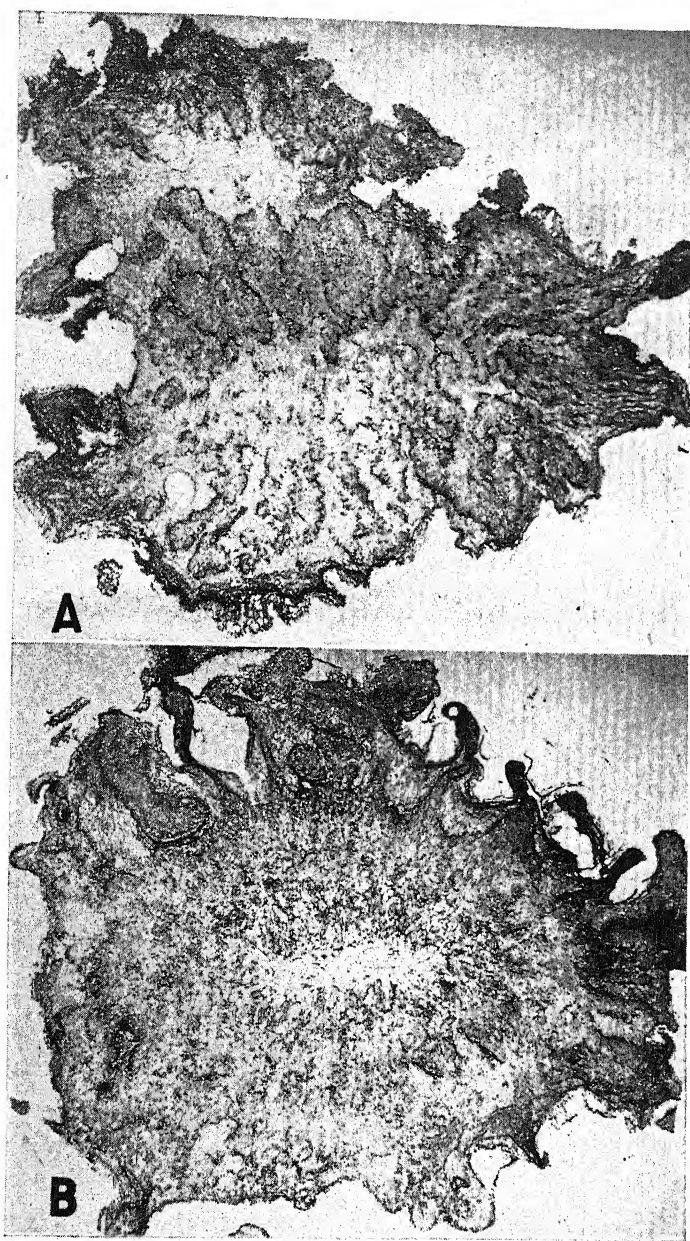


FIG. 4.—Transection of old tumor 77 days after application of lanolin mixture, and still actively developing. *A*: through tumor developed above surface of application; meristematic activity, especially at periphery, very great. All tissues from the pith. *B*: through upper level of root zone just below surface of application. Many root tips have died and been overgrown by derivatives of phloem. Pith, especially at periphery, is meristematic, but not so much so near the center.

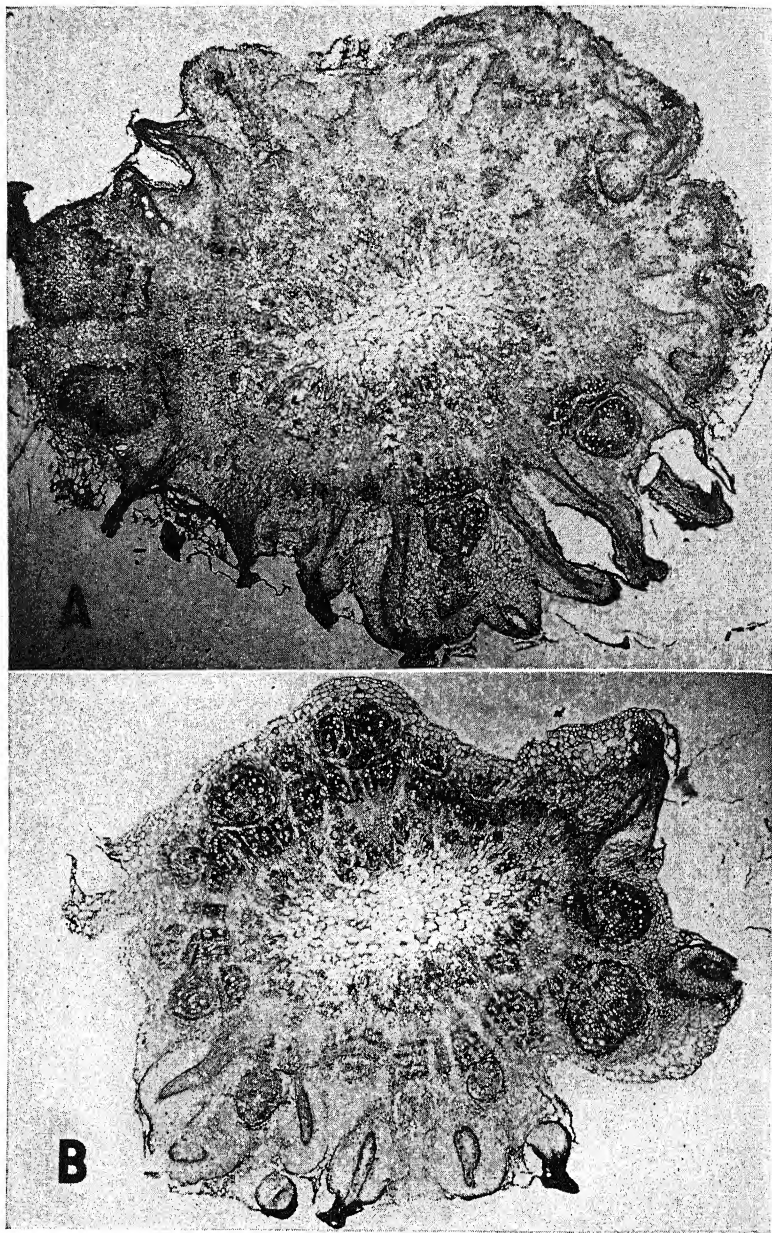


FIG. 5.—Same tumor as fig. 4. *A*: section at base of root zone. The derivatives from the phloem, the large, compact vascular masses derived either from the endodermis or phloem lie between many roots, some of which, especially their bases, are still living. Pith at center has shown little proliferation. *B*: same tumor about 1 cm. below treated surface. Bases of roots, mainly related to rays, still living. Large vascular masses over main vascular bundles are being added to by a cambium between their xylem and phloem, and frequently smaller bundles still outside these are differentiated from parenchymatous cells derived from inner cortex or endodermis.



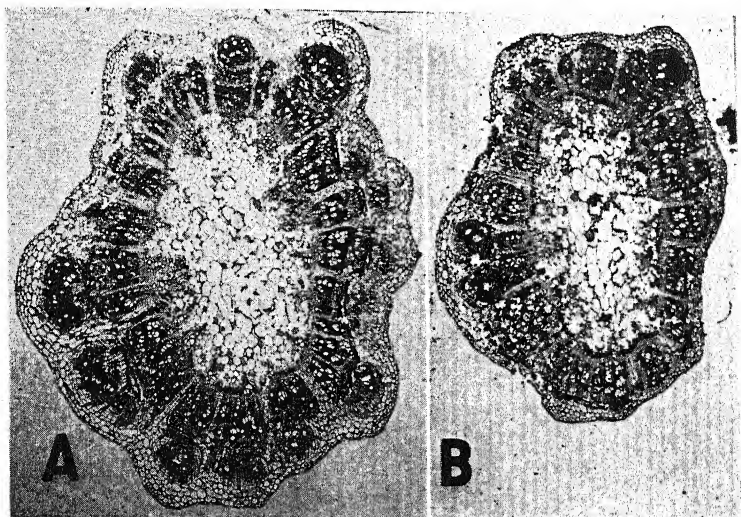


FIG. 6.—Same tumor. *A*: 3 cm. and *B*: 5 cm. below treated surface. Vascular strands derived from endodermis or phloem lie centrifugally to main bundles of stem which have undergone usual type of secondary thickening. Pith shows little or no meristematic activity. Same scale as figs. 4, 5.

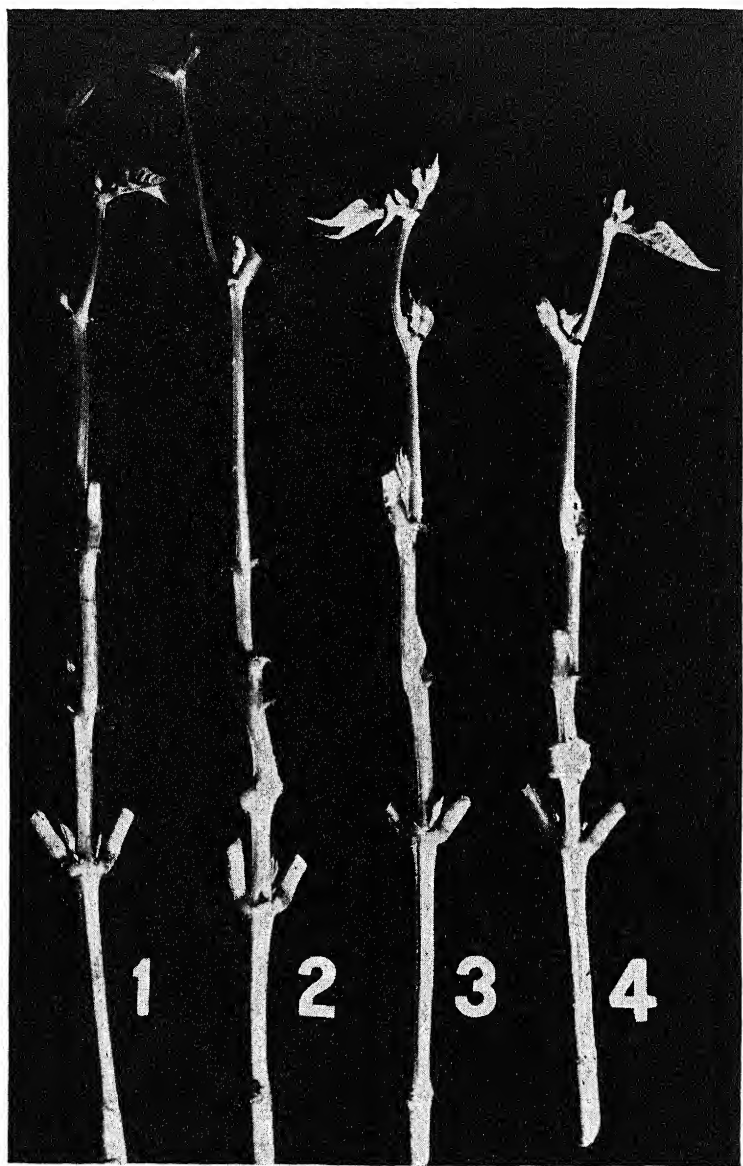


FIG. 7.—Stems 2 and 4 treated laterally with a ring of 3% indoleacetic acid lanolin mixture; 1 and 3 untreated. All 13 days after treatment. Apart from formation of the tumors and adventitious roots on treated stems, the two types were identical, with large green leaves and beginnings of flowering branches.

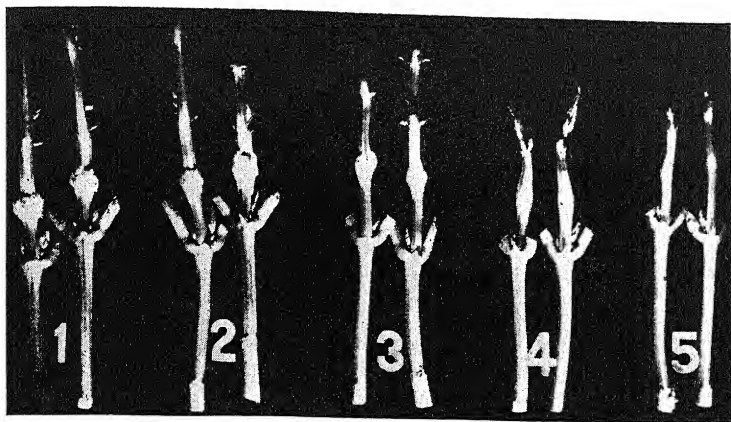


FIG. 8.—Lateral treatments around the stem, 1, thirteen days; 2, nine days; 3, six days; 4, three days; and 5, one day after application, showing rate of development of tumor. Natural size.

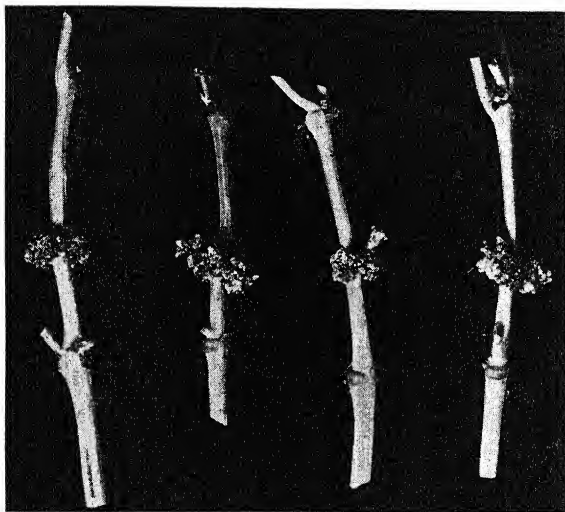


FIG. 9.—Old tumor resulting from lateral treatment 160 days after application of lanolin mixture. Tumors still green and growing slightly, although plants as a whole were greatly lacking in vigor, each having produced many flowers and seeds. Reduced about one-third.



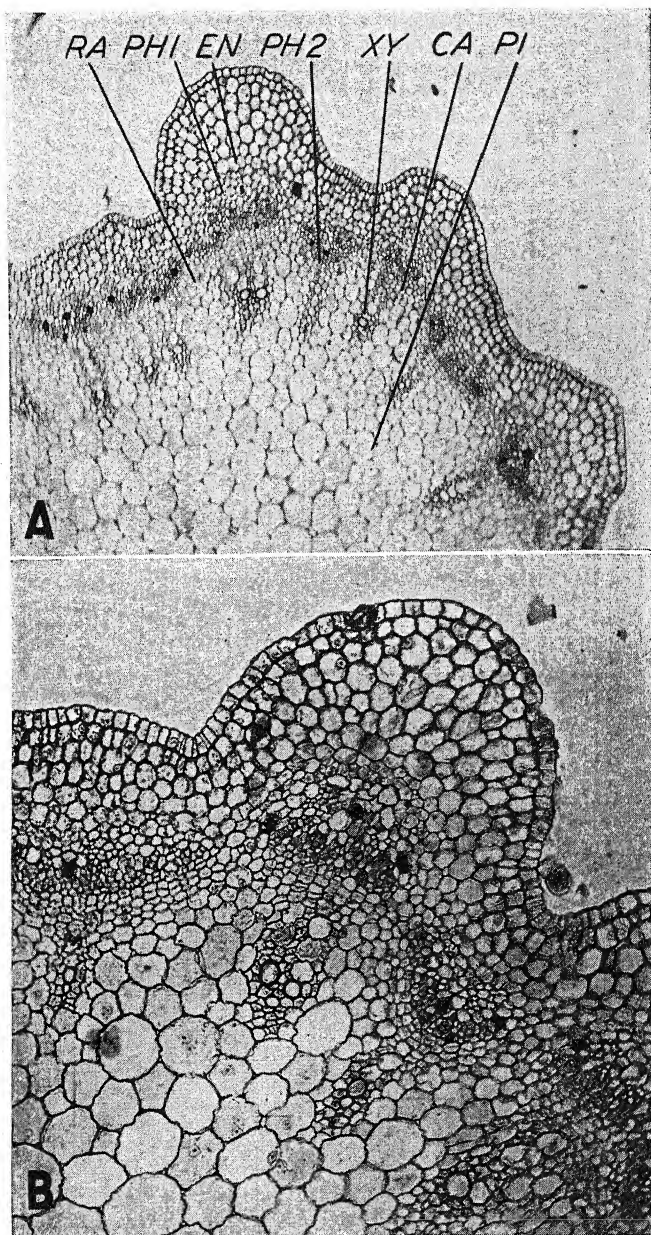


FIG. 10.—*A*: transection near top of second internode of bean at time of initial treatments, showing various tissues and stage of development (*ra*, ray; *ph<sub>1</sub>*, primary phloem; *en*, endodermis; *ph<sub>2</sub>*, secondary phloem; *xy*, xylem; *ca*, cambium; *pi*, pith). *B*: sector of same enlarged; pericyclic fibers over smaller bundles more nearly mature than those over the larger.

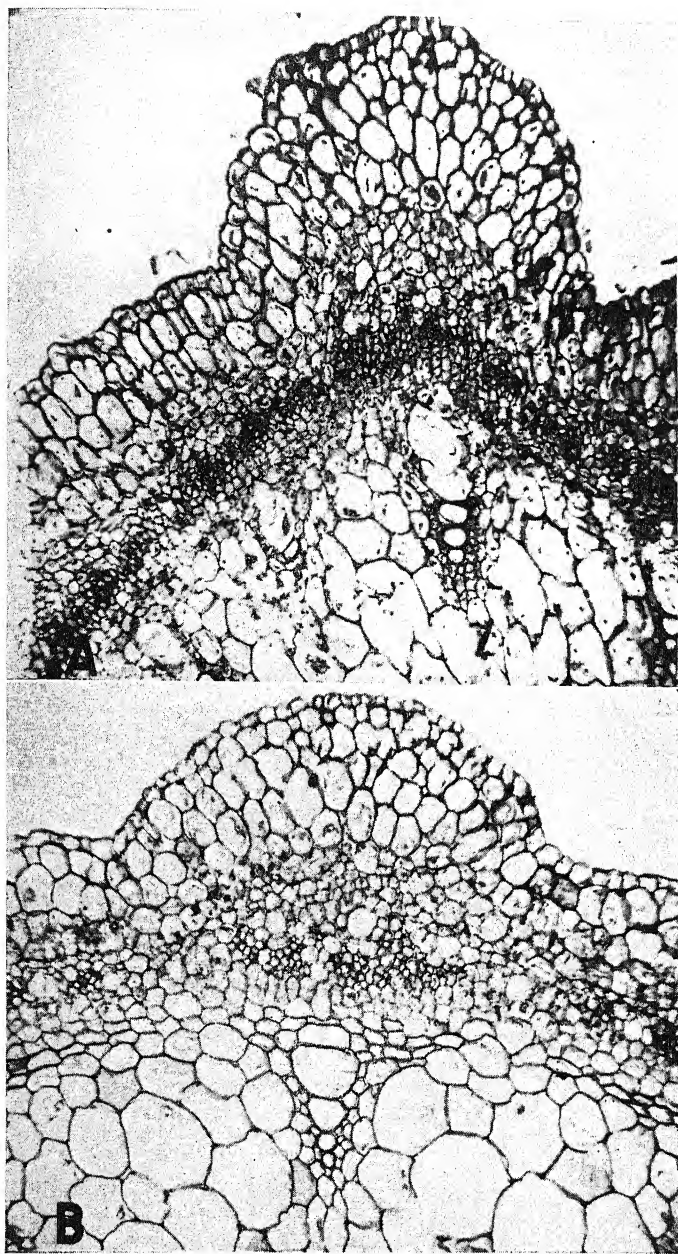


FIG. 11.—*A*: 30 hours and *B*: 48 hours after lateral treatment; sections midway of applied band of lanolin mixture. In former the cortical cells have enlarged markedly, particularly radially; in latter the endodermal cells show first tangential divisions.

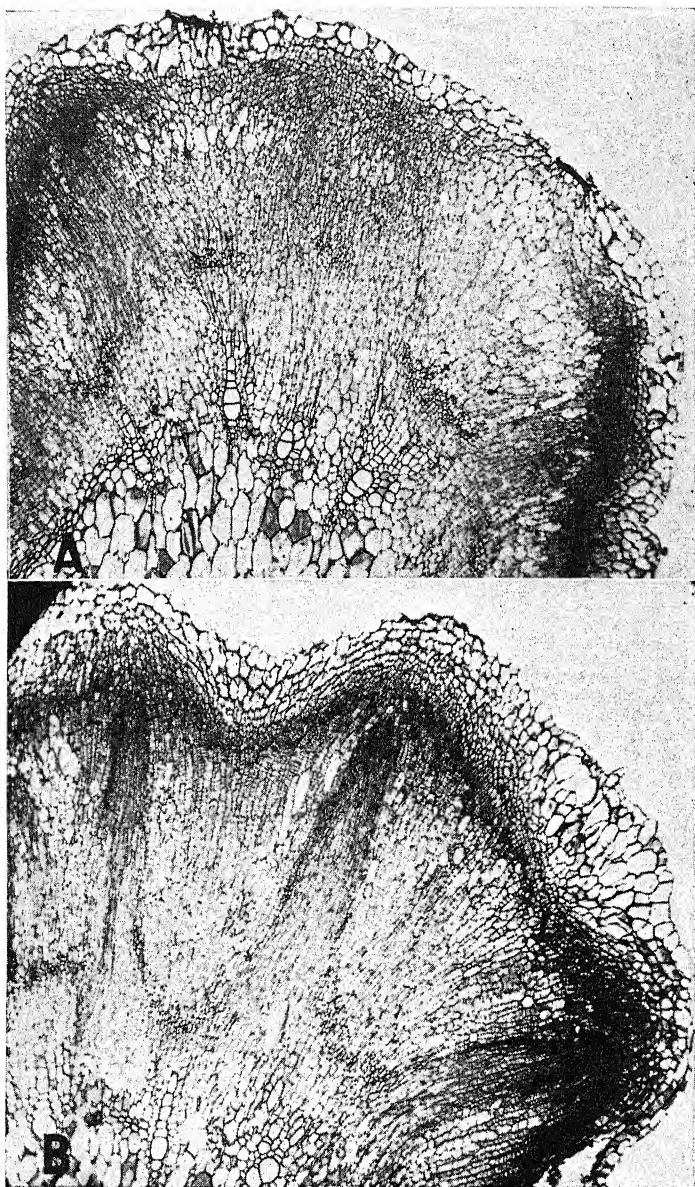


FIG. 12.—*A*: 96 hours after application; cortical, endodermal, phloem, cambial, and ray cells flanking the phloem very highly meristematic, with beginnings of roots clearly delimited in outer ray derivatives. Ray cells flanking primary xylem and pith cells have not responded meristematically. *B*: 114 hours after application; roots clearly marked; inner ray cells and pith show virtually no meristematic activity but have progressed toward maturity.



FIG. 13.—Longitudinal section 136 hours after application; narrow band of lanolin mixture applied about midway of distance now showing roots. As usual there is as much response above line of application as below it, with reference to formation of roots, but formation of vascular strands derived from phloic and endodermal cells extends appreciably farther down stem than above it. In no case have tissue changes or tumor formation extended either as far up or down the stem as when lanolin mixture is applied to terminal cut surface, unless grown in saturated atmosphere or in darkness.

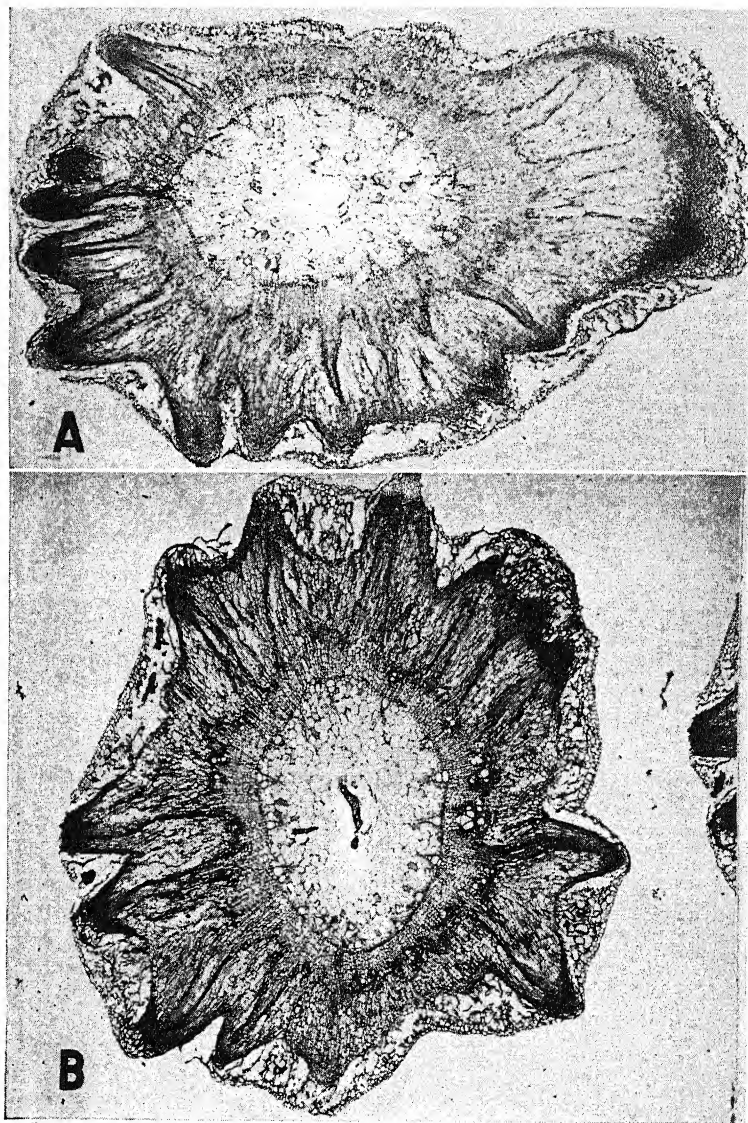


FIG. 14.—*A*: 9 days and *B*: 23 days after application, section through line of application. Pith at center has matured and begun disintegration in contrast to behavior of pith in root zone when mixture is applied to apical cut surface (fig. 5). Comparatively little meristematic activity.





FIG. 15.—Section 66 days after lateral application. Parenchymatous tissues of vascular strands between adventitious roots and those of proliferated phloem are meristematic. These may continue proliferation over a long period of time, finally producing large tubercles and flanges of vascular and parenchymatous tissue which overgrow the adventitious roots (fig. 16). Pith not meristematic.

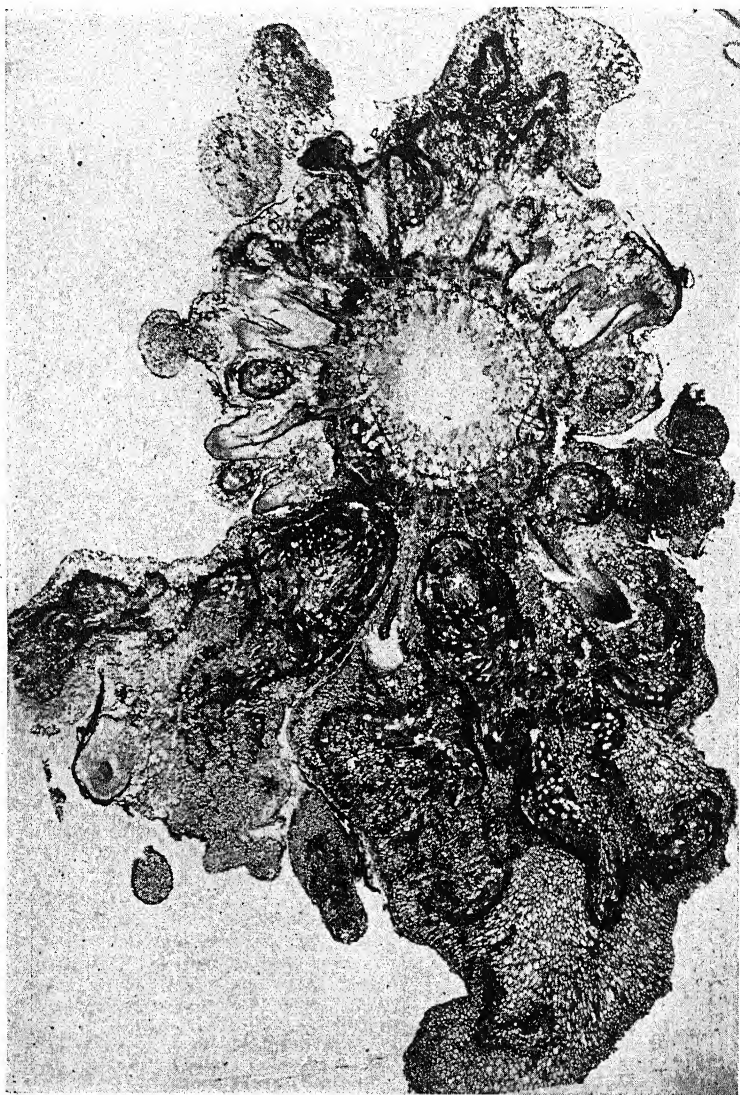


FIG. 16.—Transection through tumor showing several stages in development of masses of tissue from derivatives of phloem and derived endodermal tissues, in places completely enveloping the early formed adventitious roots. Other roots may be formed indiscriminately among these proliferating tissues.

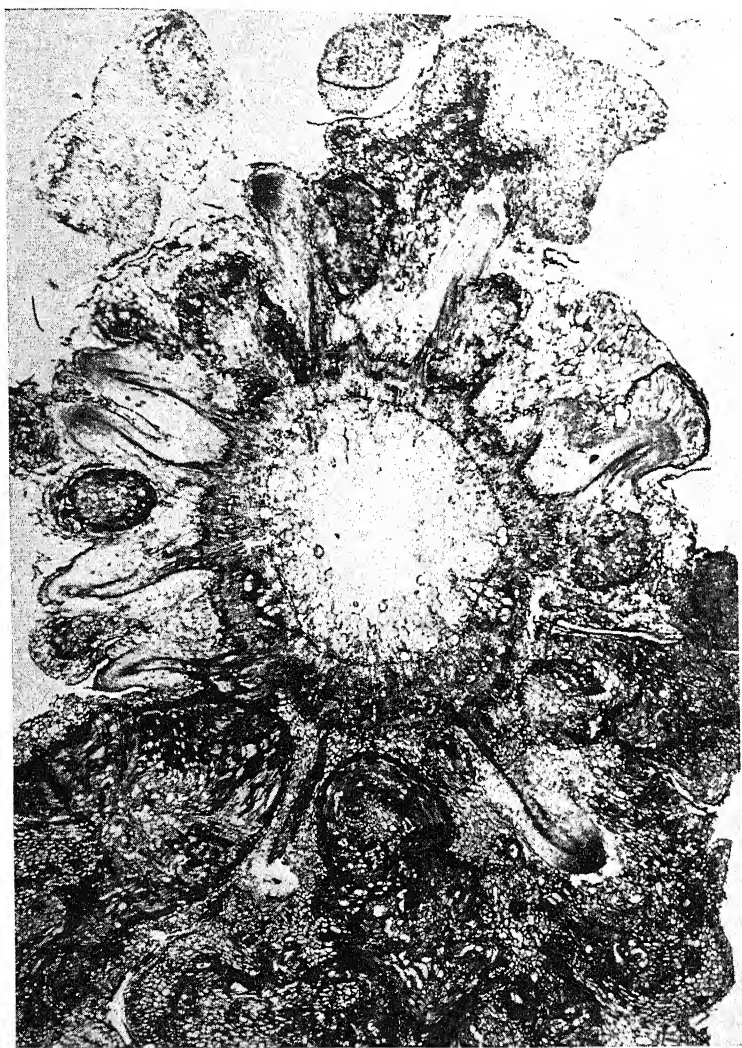


FIG. 17.—Same as fig. 16, enlarged. Cells of rays flanking primary xylem and of pith are non-meristematic and no derivatives from them have contributed to the large irregular masses of tumor.



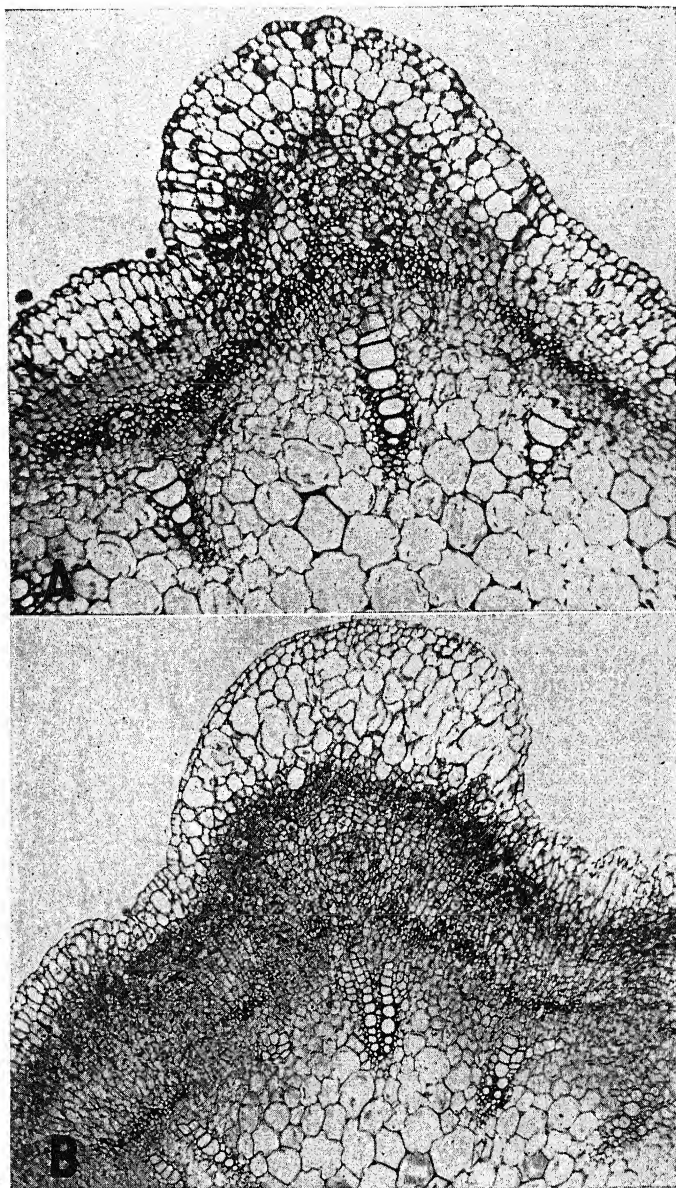


FIG. 18.—Transection of decapitated stem slightly below surface to which 1.5% indolebutyric acid in lanolin has been applied. *A*: 48 hours after treatment; inner cortical, endodermal, phloem, and ray cells actively meristematic. *B*: 72 hours after treatment; marked activity of endodermis. Vascular strands in proliferated phloem are being differentiated, as well as root primordia in rays, and, at places, from the endodermal derivatives. Cells of pith slightly meristematic.

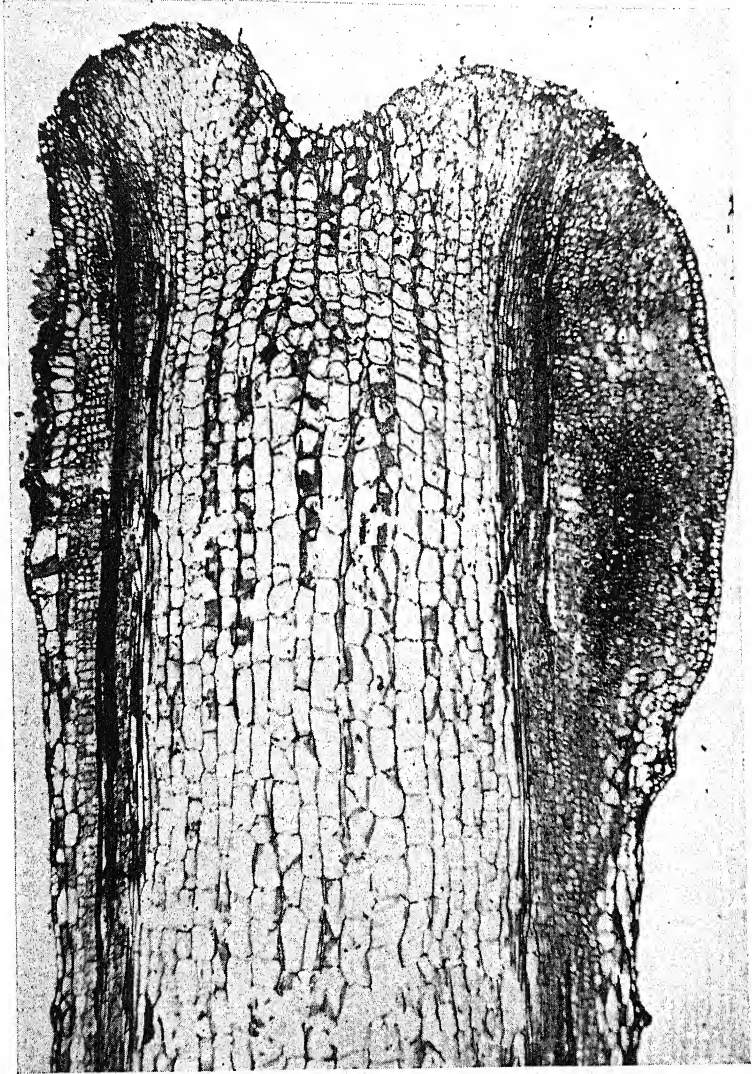


FIG. 19.—Longitudinal median section of developing tumor 96 hours after application of indolebutyric mixture. Cells of endodermis have proliferated markedly many cell layers below treated surface. Near top the cortical cells are active out to the epidermis. Those of pith are also active, but for far less distance down the stem.

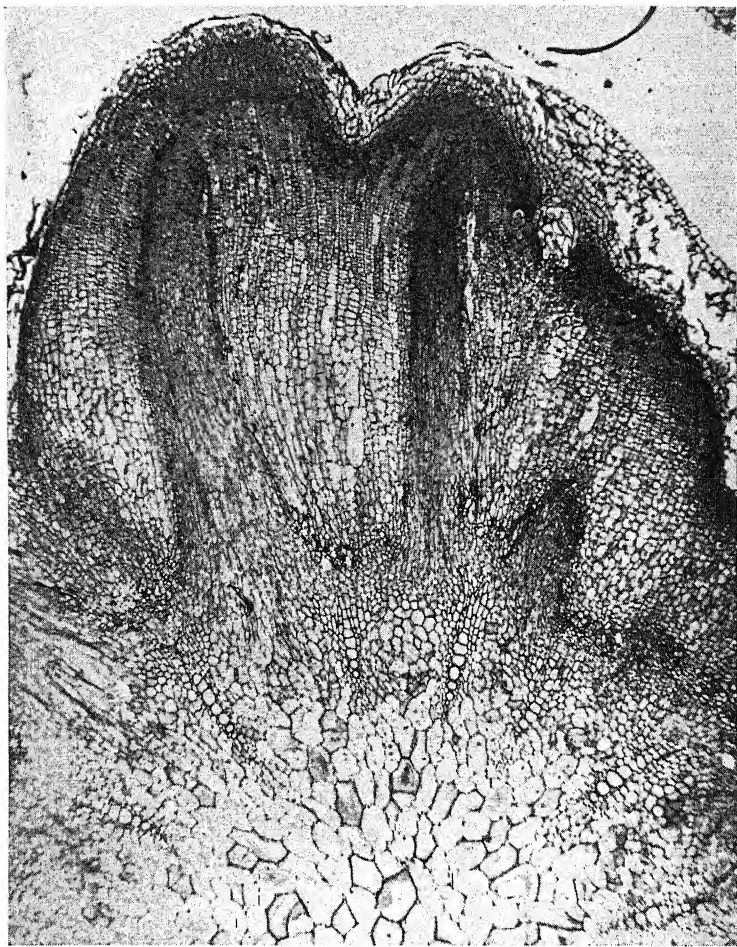


FIG. 20.—Transverse section through zone of adventitious roots 112 hours after treatment of cut surface with indolebutyric mixture. Cells of ray and some cells of pith are active, but less so than is usually true in stems treated with a 3% indoleacetic lanolin mixture.

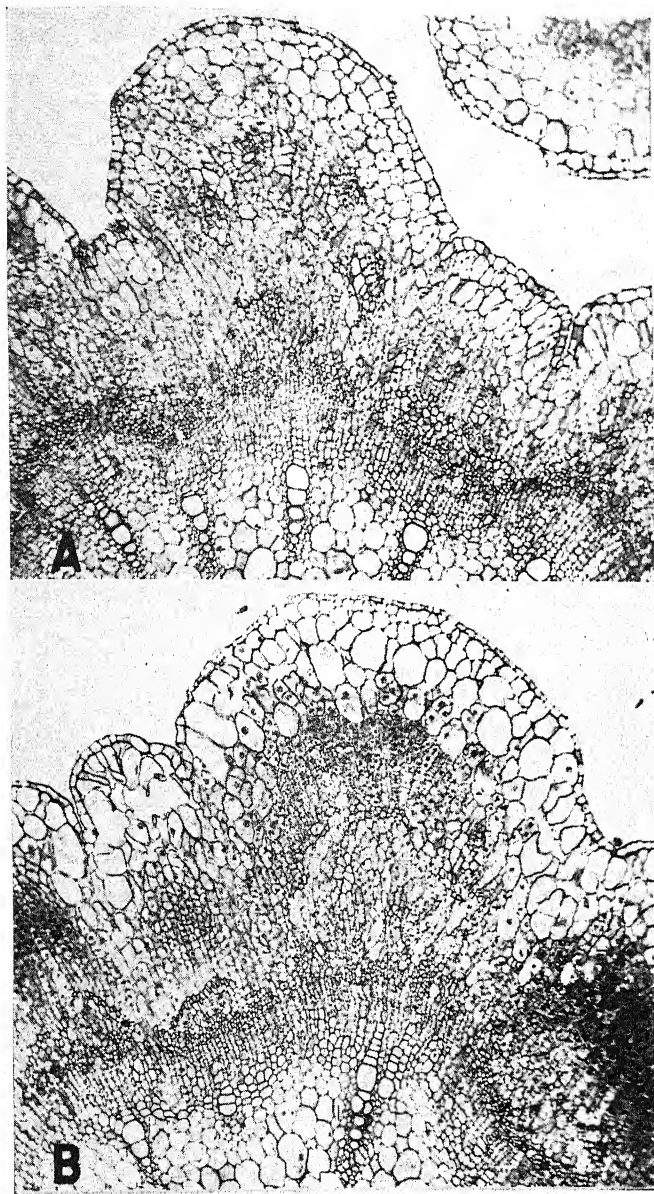


FIG. 21.—Transverse section of developing tumor 96 hours after treatment of cut surface with indolebutyric mixture. *A*: developing vascular strands from endodermal derivatives. *B*: another area in same section; root primordia being differentiated from endodermal derivatives over a phloem area.

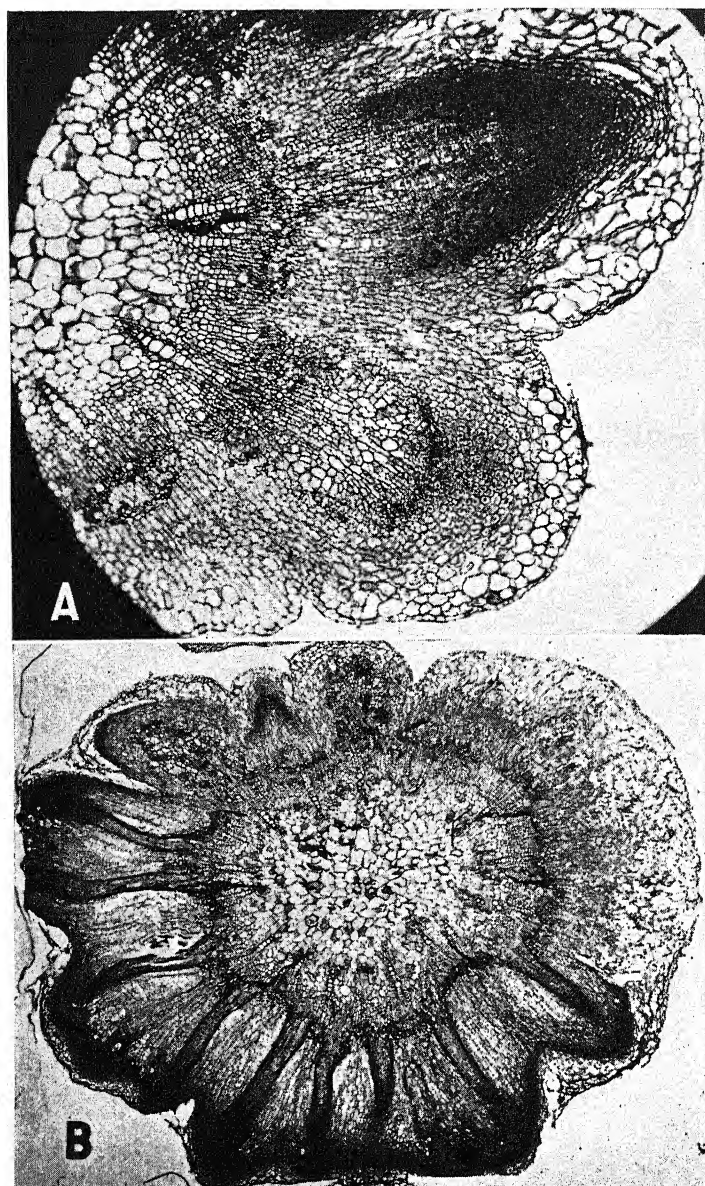


FIG. 22.—Transverse sections of tumor 162 hours after application of indolebutyric mixture. *A*: two bundles showing developments as in fig. 21*A*, *B*. Over one group a root initial has developed largely from endodermal derivatives; over the other a vascular strand is being differentiated partly from phloem derivatives, partly from endodermal derivatives, the latter still highly meristematic. In some cases root primordia arise from these exterior patches of endodermal cells. *B*: section through upper zone of adventitious roots differentiated mainly from ray cells, but some of them lie exterior to the phloem and are mainly endodermal in origin.



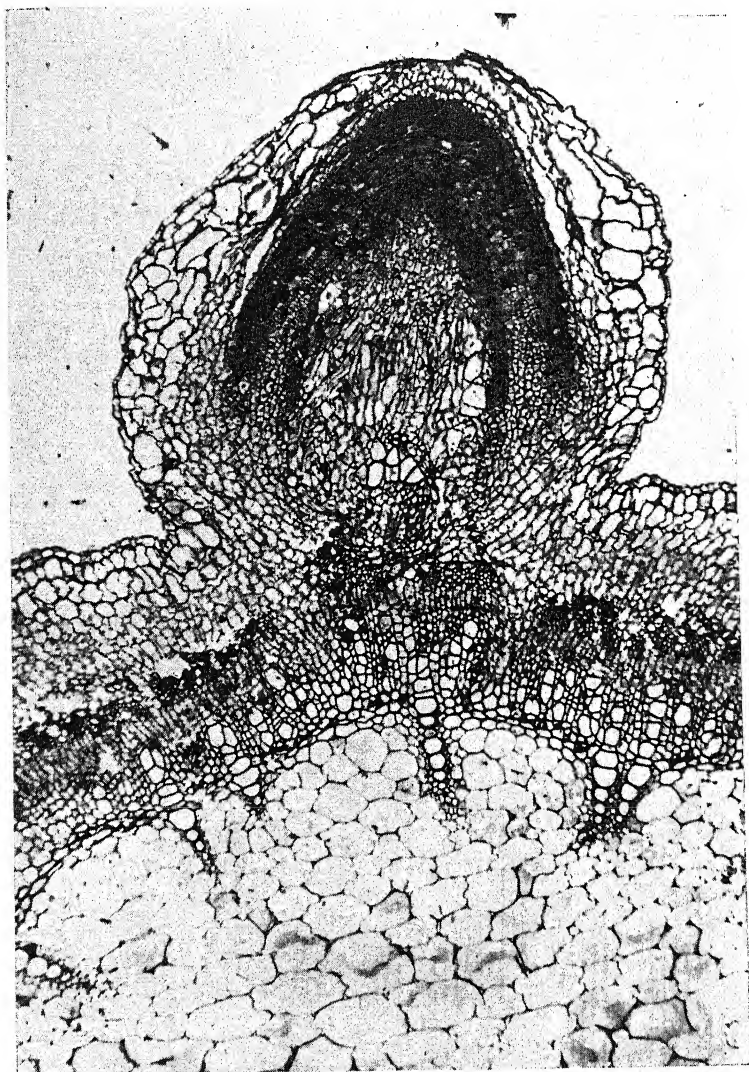


FIG. 23.—Transection of stem about 7 mm. below surface treated with indolebutyric mixture, and below main zone of adventitious roots nearer surface. Roots developed at lower levels generally occupy positions over the phloem instead of in the rays. Same age as fig. 22.

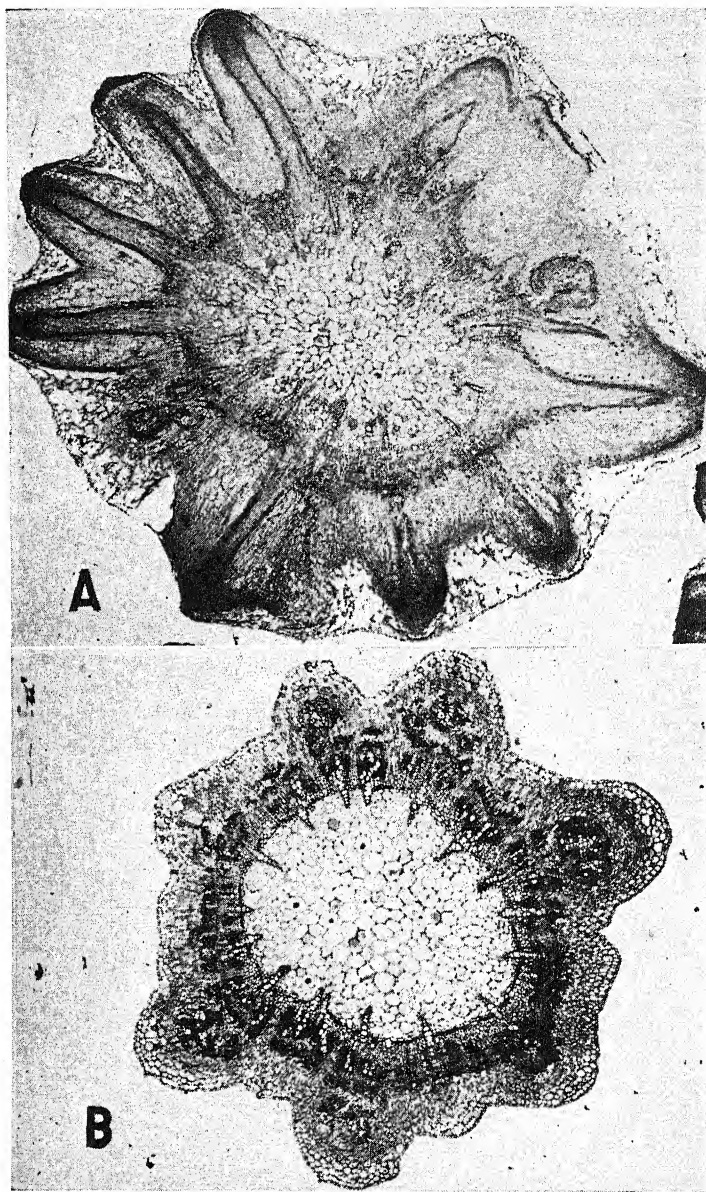


FIG. 24.—Section 216 hours after treatment of cut surface with indolebutyric acid lanolin mixture. *A*: through root zone near treated surface. *B*: about 2 cm. below. Outer vascular strands derived principally from endodermis over principal vascular bundles of stem. Other portions of stem have developed much as in an untreated stem.



FIG. 25.—Longitudinal section of apical tumor 216 hours after treatment with 1.5% indolebutyric acid in lanolin. Dark patches near top indicate level of treated surface. Above these the vascular apical tumor, derived mainly from pith derivatives, has begun to form. It develops almost exactly as do similar tumors resulting from application of 3% indoleacetic acid in lanolin (fig. 3). Below treated surface is an extensive root zone. The uppermost of these roots are generally initiated from endodermal and ray derivatives, sometimes two or more rays being involved in initiation of a single large root; lowermost ones generally initiated from cells over a proliferated phloem group of main vascular bundles of stem. Such a root is at the lower right.



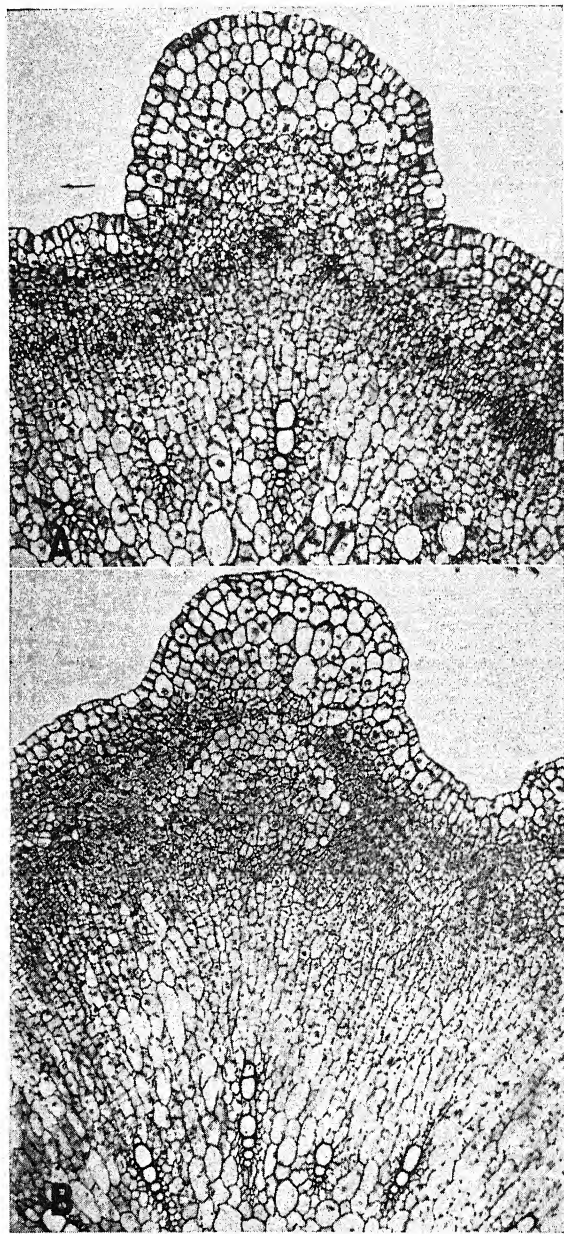


FIG. 26.—Transsections of stems decapitated and treated with 0.5% naphthalene-acetic acid in lanolin. *A*: 48 hours after treatment; endodermal, phloic, cambial, and ray cells meristematic. *B*: 72 hours after treatment; outer cortical cells meristematic as well as those of endodermis. The meristematic activity of other regions except epidermis, pericycle, and pith is very great. Root initials evident in rays.

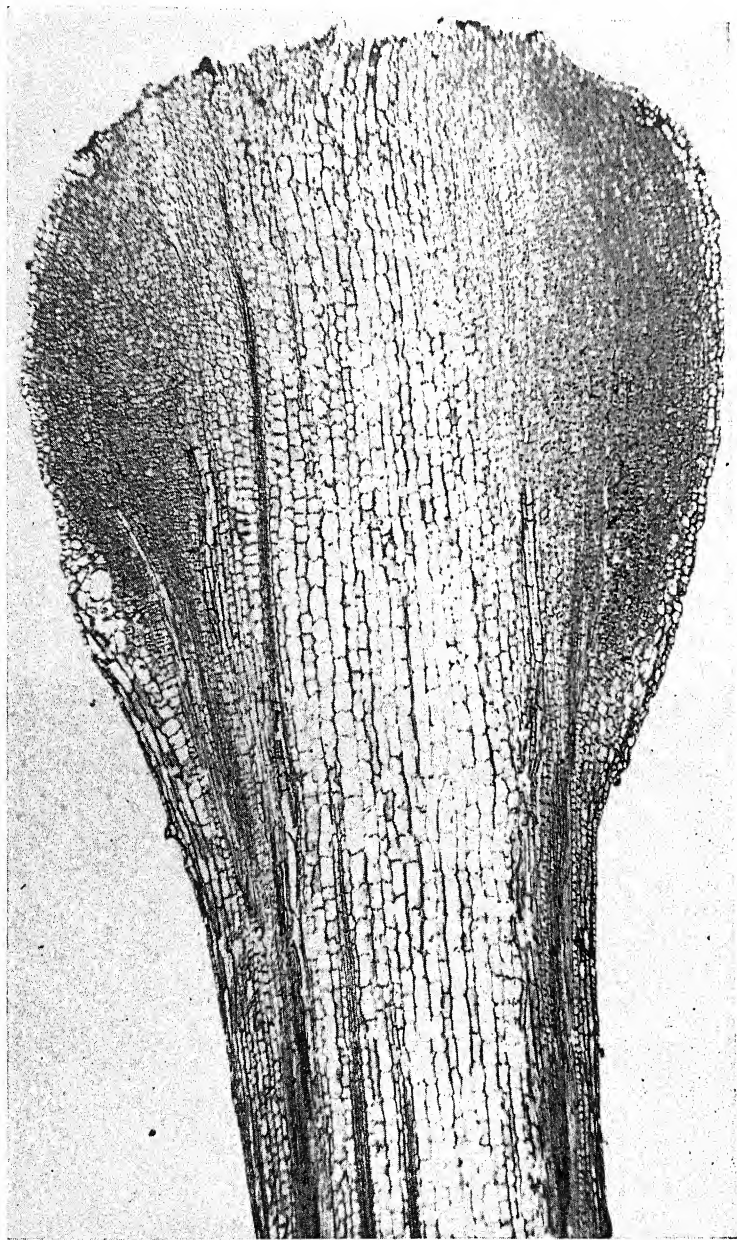


FIG. 27.—Longitudinal median section of apical tumor 72 hours after treatment of cut surface with 0.5% naphthaleneacetic acid. Activity of cortical, endodermal, phloem, ray, and cambial cells extends many cell layers down the stem. Pith shows very little meristematic activity except immediately below treated surface (fig. 29).

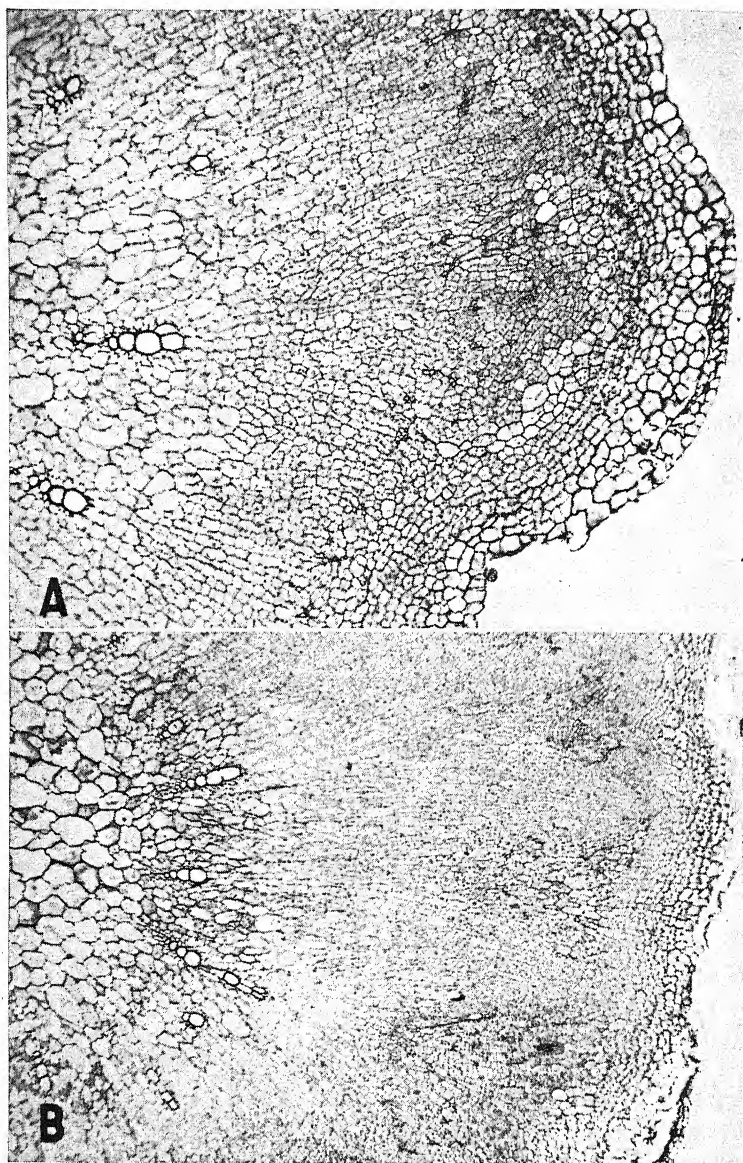


FIG. 28.—*A*: 96 hours after decapitation and treatment of surface with 0.5% naphthaleneacetic acid mixture. In addition to other tissues, some of pericyclic cells are meristematic. Pith shows almost no activity. Section about  $150\ \mu$  below treated surface. *B*: similar section 120 hours after treatment. Root initials in rays clearly delimited.



FIG. 29.—Transverse section of decapitated stem treated with 0.5% naphthalene-acetic lanolin mixture,  $80\mu$  below treated surface. Some of pith cells dead and disrupted, others highly meristematic and from these vascular masses are differentiating. Such activity is limited to layers near treated surface.



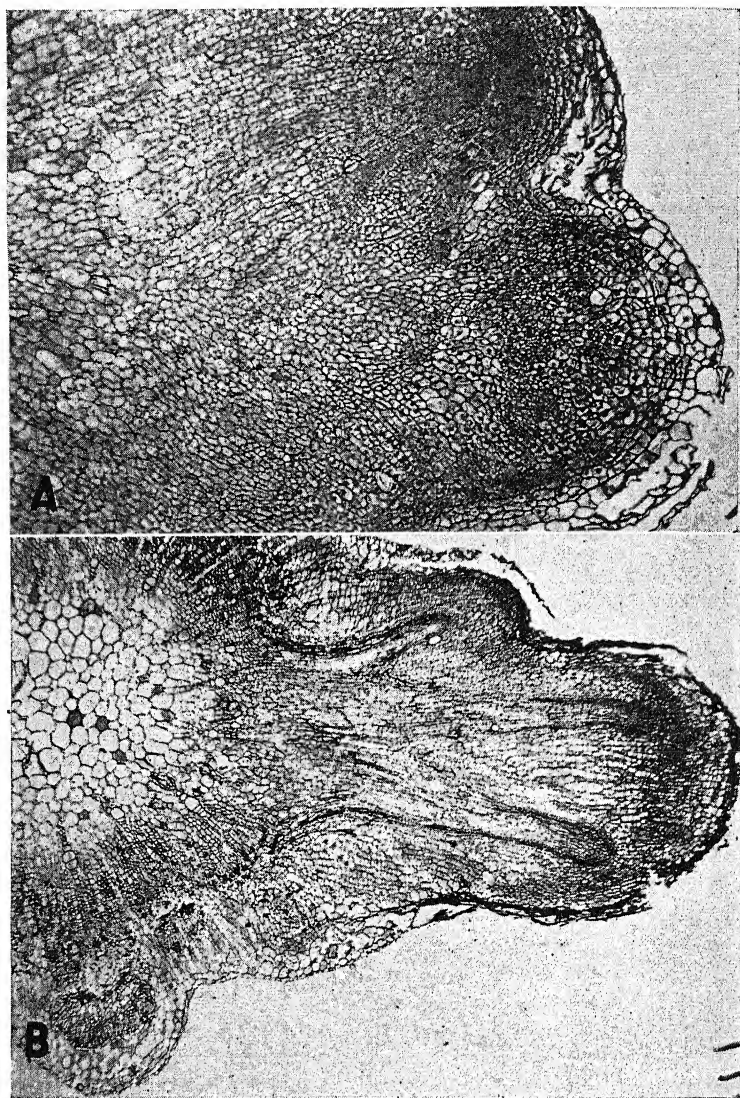


FIG. 30.—Two sections about 1 cm. below surface treated with 0.5% naphthalene-acetic lanolin mixture. *A*: 168 hours after treatment. A root is differentiating in a ray; over the phloem of an adjacent bundle the endodermis and other cortical cells are highly meristematic. *B*: similar situation after 32 days of development. A root complex has involved at least three adjacent vascular bundles. Structures such as these frequently occur in a circle around the stem, over some of vascular bundles, about 1 cm. below treated surface (fig. 32*A*).

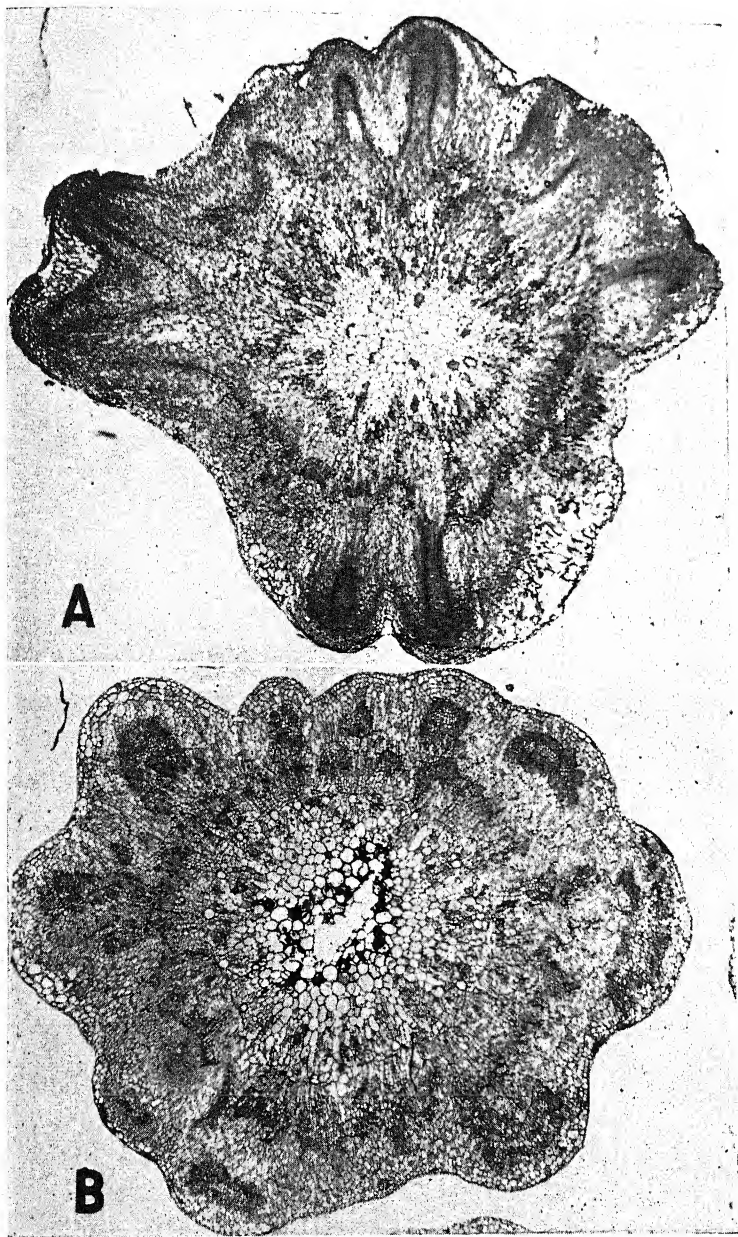


FIG. 31.—Section 32 days after decapitation and treatment of cut surface with 0.5 % naphthaleneacetic acid lanolin mixture. *A*: through root zone about 0.5 cm. below treated surface. *B*: just below root zone about 1 cm. below treated surface. Pith is meristematic immediately adjacent to primary xylem vessels. Over principal vascular bundles vascular strands in multiple series up to three or more, separated by parenchymatous cells, have differentiated mainly from endodermal and other cortical derivatives. Cortical cells generally more active than those at same level from treated surface of tumors resulting from 3% indoleacetic acid lanolin mixture or 1.5% indolebutyric acid lanolin mixture.

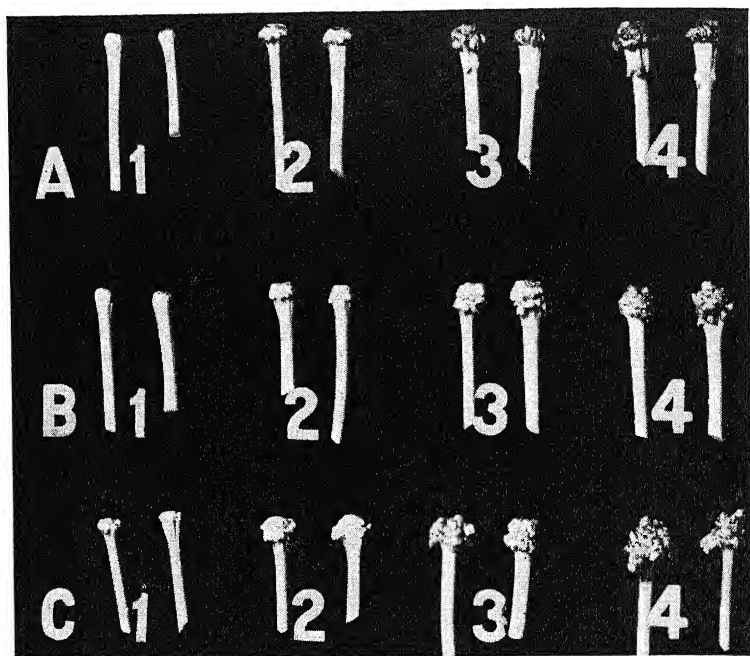


FIG. 32.—A: apical tumors resulting from application of 1.5% naphthaleneacetic acid; B: 1.5% indolebutyric acid; C: 1.5% indoleacetic acid, all in lanolin mixture. 1, 85 hours; 2, 7 days; 3, 14 days; 4, 21 days after application. Thickening of stem for about 1 cm. below surface of application is characteristic for tumors resulting from naphthaleneacetic acid in our cultures. Such tumors have never produced large apical tumors from proliferated pith and soon cease apical expansion. Development of large, vascular, apical tumors from pith characterizes effects resulting from indoleacetic and indolebutyric acid. Those here are just beginning to develop and during subsequent weeks would have attained several times their present size and height.

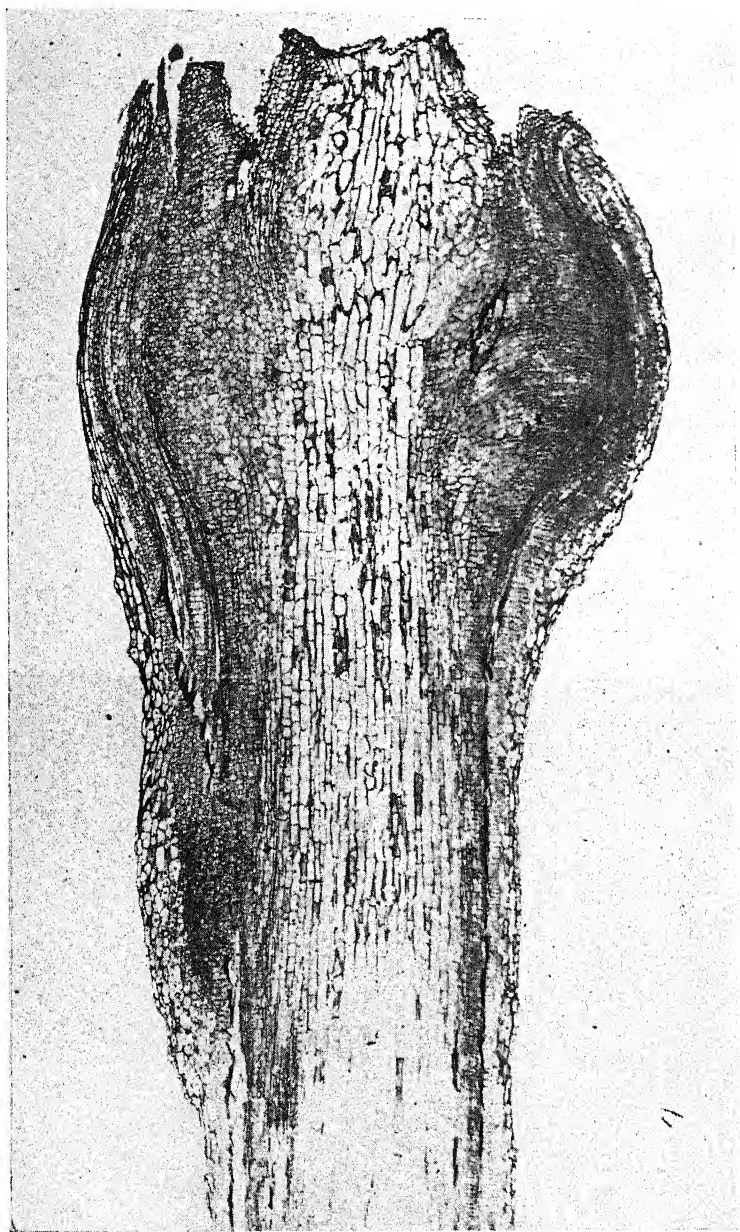


FIG. 33.—Four-day-old apical tumor resulting from application of 1.5% naphthaleneacetic acid in lanolin. Tissues near surface have been killed by this concentration, but several cell layers distant the responses are characteristic for this substance used in lower concentration (fig. 27).



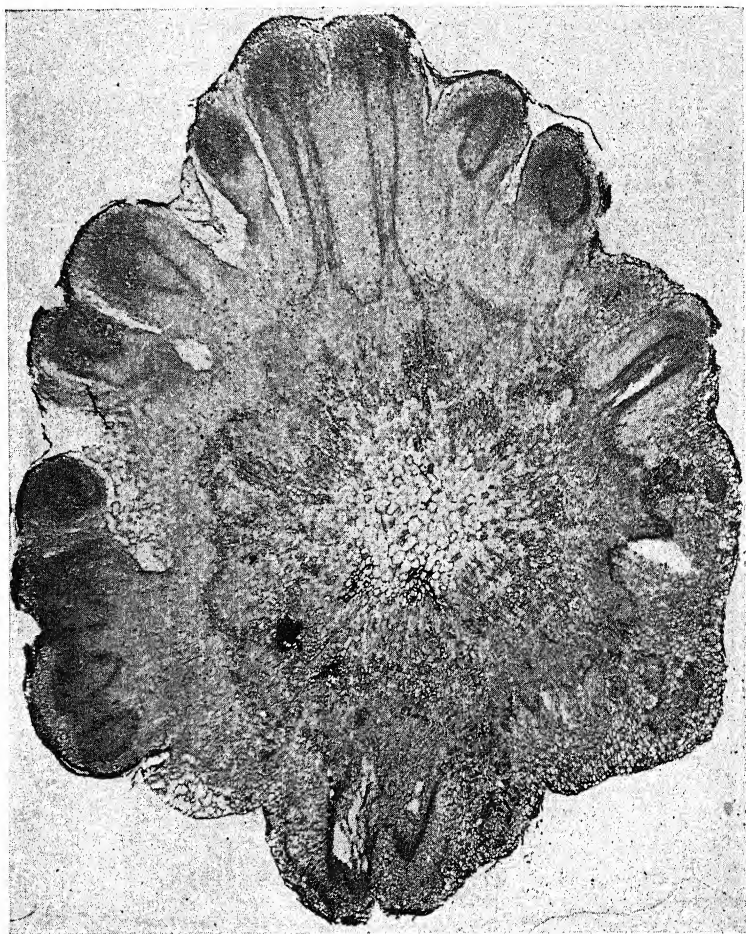


FIG. 34.—Section through root zone of apical tumor resulting from application of 1.5 % naphthaleneacetic acid lanolin mixture 21 days after application. It is more closely similar to tumors produced from 0.5% mixture of same substance than to tumors resulting from other two substances employed.

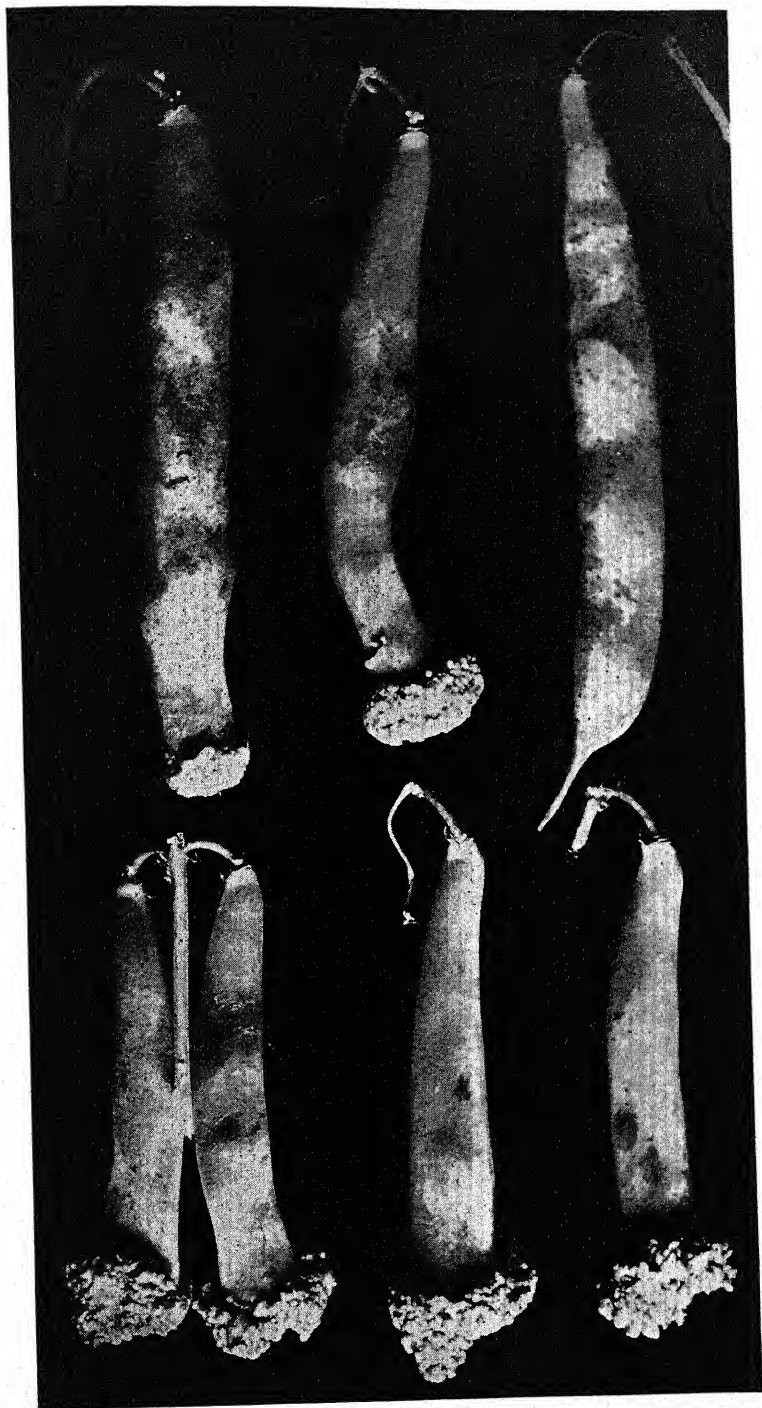


FIG. 35.—Tumors on bean pods 33 days after excising tip and treating with 3% indoleacetic acid lanolin mixture. In pod at upper left three seeds are maturing; at upper center, one seed; apical tumors smaller than those on pods in which seeds have aborted (lower row). Pod at upper right untreated. Tissues proliferate markedly only close to surface of application. Natural size.



FIG. 36.—Tumors on bean pods 33 days after slightly abrading epidermis at adaxial suture and applying along it a thin layer of 3% indoleacetic acid in lanolin. All seeds have ceased to develop. Proliferation has occurred close to surface of application only. Reduced about one-third.

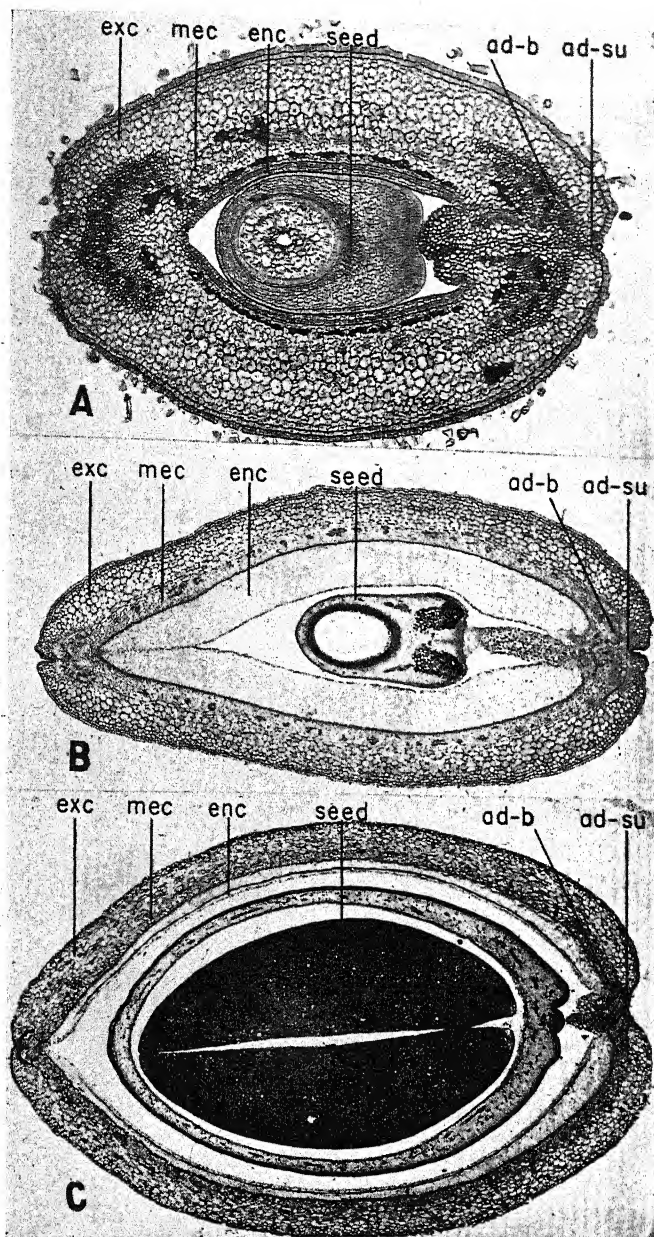


FIG. 37.—Transverse section of Red Kidney bean pods, untreated, through developing seed. *A*: shortly after fertilization; petals withered, entire pod about 1 cm. long, 1.5 mm. suture to suture diameter. *B*: several days later; pod about 5 cm. long, 5 mm. suture to suture diameter. Nearly all treatments of pods were made at this stage. *C*: nearly mature pod 12–14 cm. long, 1.5 cm. suture to suture diameter (*exc*, exocarp, non-vascular and parenchymatous except for layer of fibrous cells below epidermis; *mec*, mesocarp including vascular bundles, those at adaxial and abaxial sutures limited outwardly by fibers; *enc*, endocarp, parenchymatous and non-vascular, derived wholly or mainly as proliferation of inner epidermis; *seed*, seed; *ad b*, adaxial bundles, capped with fibers; *ad su*, adaxial suture). Various degrees of enlargement.

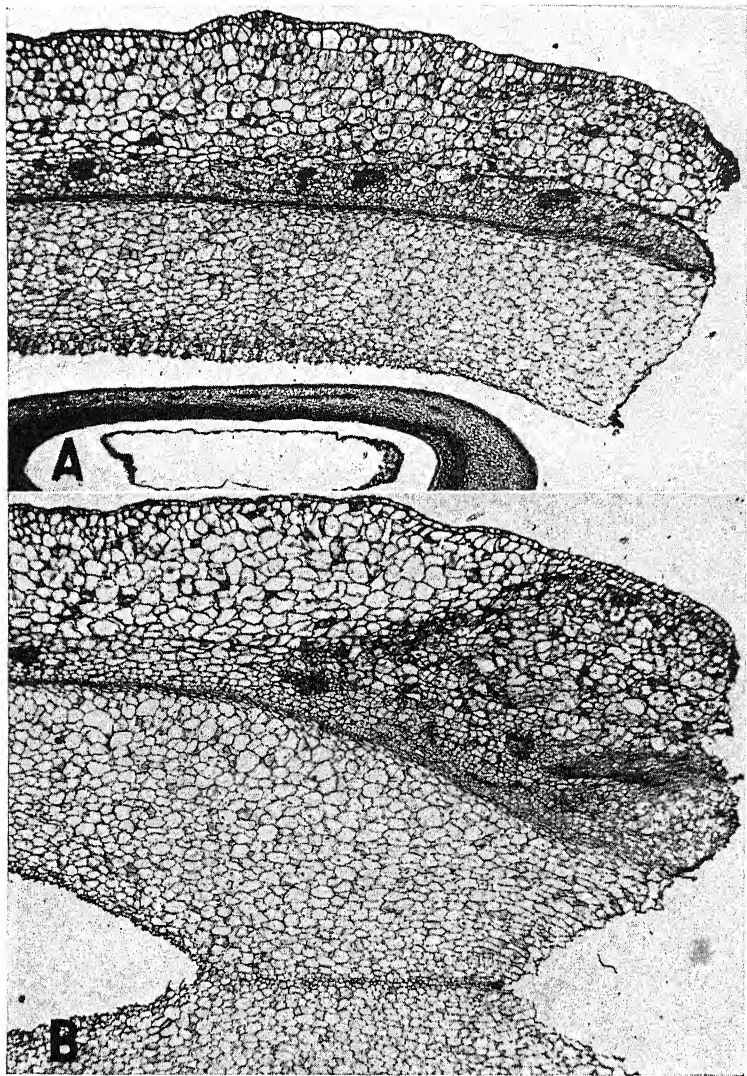


FIG. 38.—Pods 96 hours after having been cut off and treated with 3% indoleacetic acid in lanolin at cut surface. Sections cut longitudinally. *A*: pod with slight proliferation, limited mainly to parenchymatous cells of mesocarp. *B*: more usual condition, with marked proliferation of exocarp, mesocarp, and endocarp near surface of application.



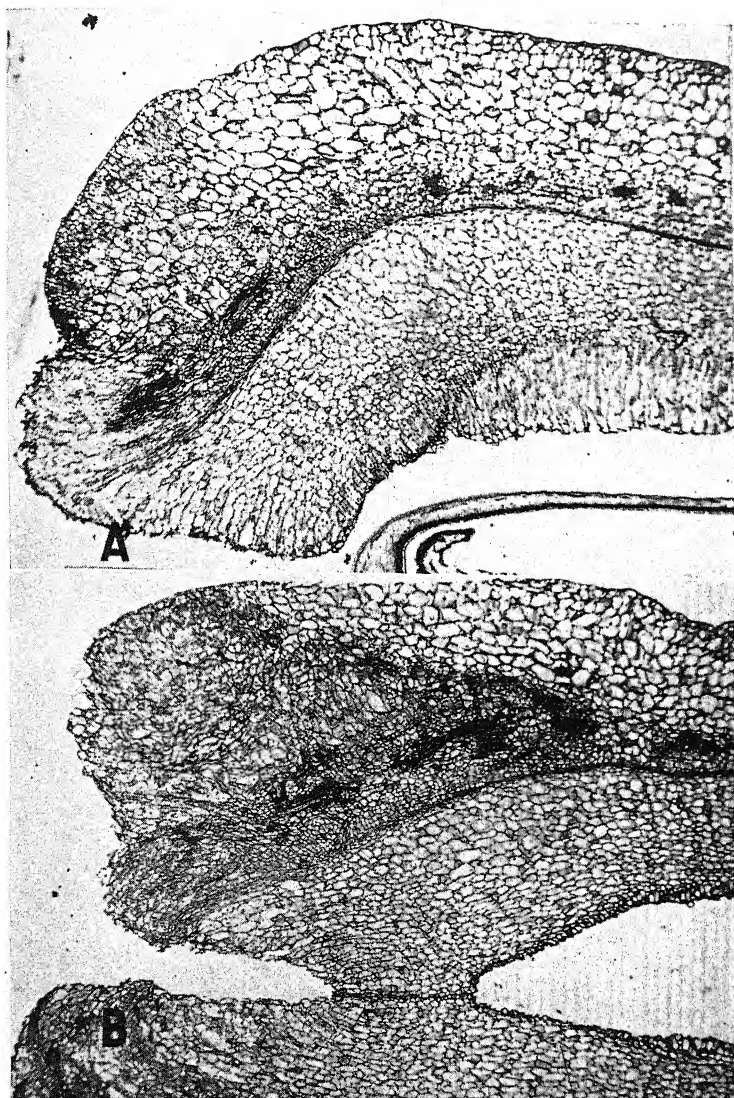


FIG. 39.—Similarly treated pods. *A*: 5 days after treatment. Some of proliferated cells of exocarp differentiating as vascular elements as are those of mesocarp, eliminating a clear line of demarcation between them. Endocarpic cells more highly meristematic near surface, but less active than other regions. *B*: 6 days after treatment, showing further progress in meristematic activity and differentiation of tissues. Derivatives of exocarp and mesocarp closing over endocarp.

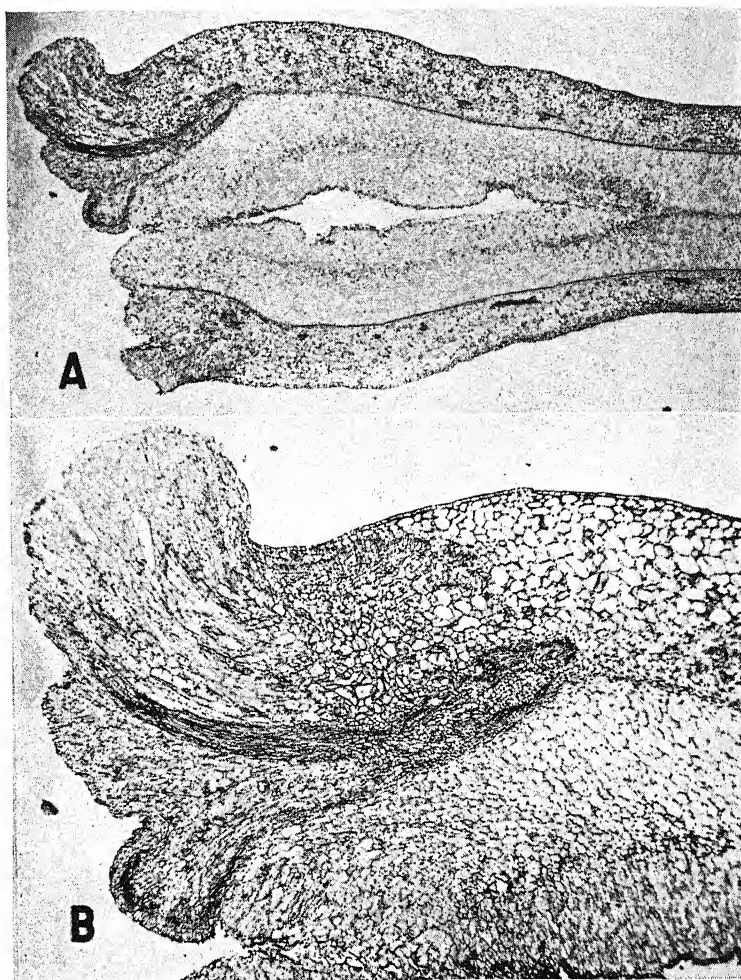


FIG. 40.—Similar pod 9 days after treatment. *B*: same as *A*, enlarged. Response of tissues obvious. Greatest and most active response is by exocarpic cells and that near surface of application. Marked response here of endocarpic cells at considerable distance from treated surface is probably result of accumulation of exudate in cavity of pod following amputation of tip and diffusion of indoleacetic acid through this over a large surface. No trace of vascular structures in endocarp.

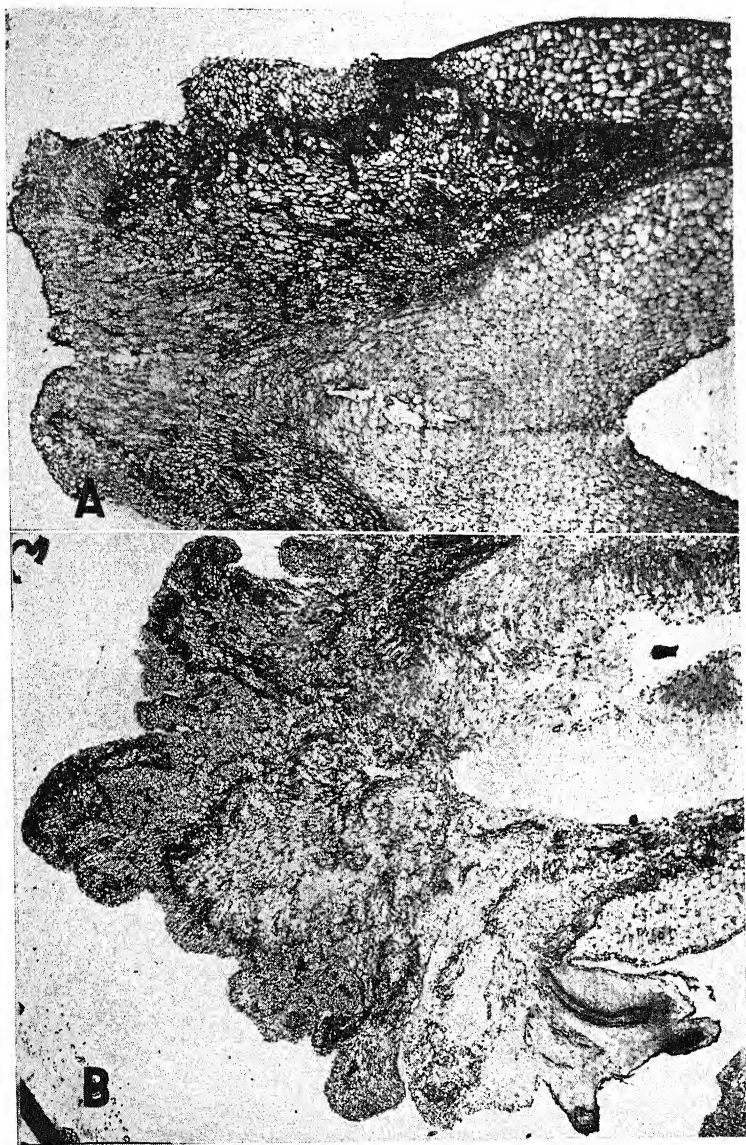


FIG. 41.—Similarly treated pods. *A*: 12 days, *B*: 20 days after treatment. Vascular strands, parenchymatous masses, and roots are differentiated from exocarpic and mesocarpic derivatives. Proliferation continues most markedly near surface and occurs irregularly, particularly under small films or masses of lanolin mixture which are carried out by proliferating and developing cells beneath them, thus making outer portions of tumor rough and tuberculated instead of smooth.



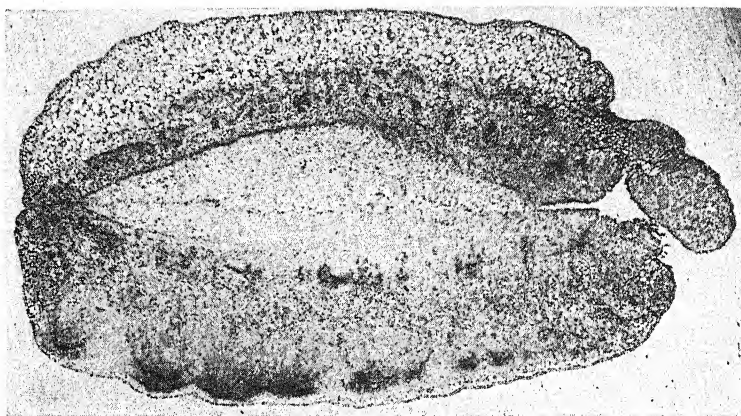


FIG. 42.—Pod with tip cut off, then treated apically. Transverse section 7 days after and practically at level of treatment, but just above adaxial bundles which would be at the right. Primordia of a number of roots differentiating from exocarpic derivatives.

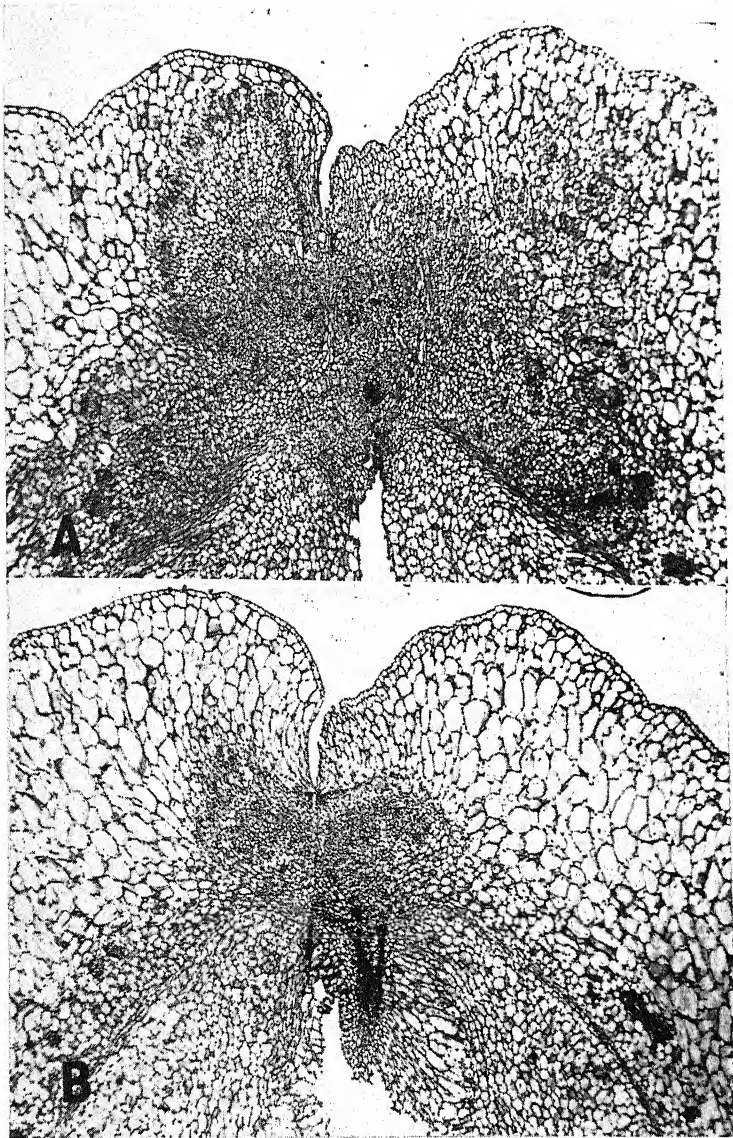


FIG. 43.—Same pod as fig. 42. *A*: about  $300\ \mu$  below level of fig. 42. Exocarpic cells proliferating and differentiating; those of mesocarp show almost no response. *B*:  $150\ \mu$  below *A*. Slight activity in exocarp only. In marked contrast to apically treated stems, cells of vascular bundles and those in their immediate vicinity show very little meristematic activity at other than very short distances from treated surface. Those derivatives away from lanolin mixture mature into vascular elements, although some remain as meristematic masses scattered between them. Those near lanolin mixture continue actively meristematic (figs. 50–53).

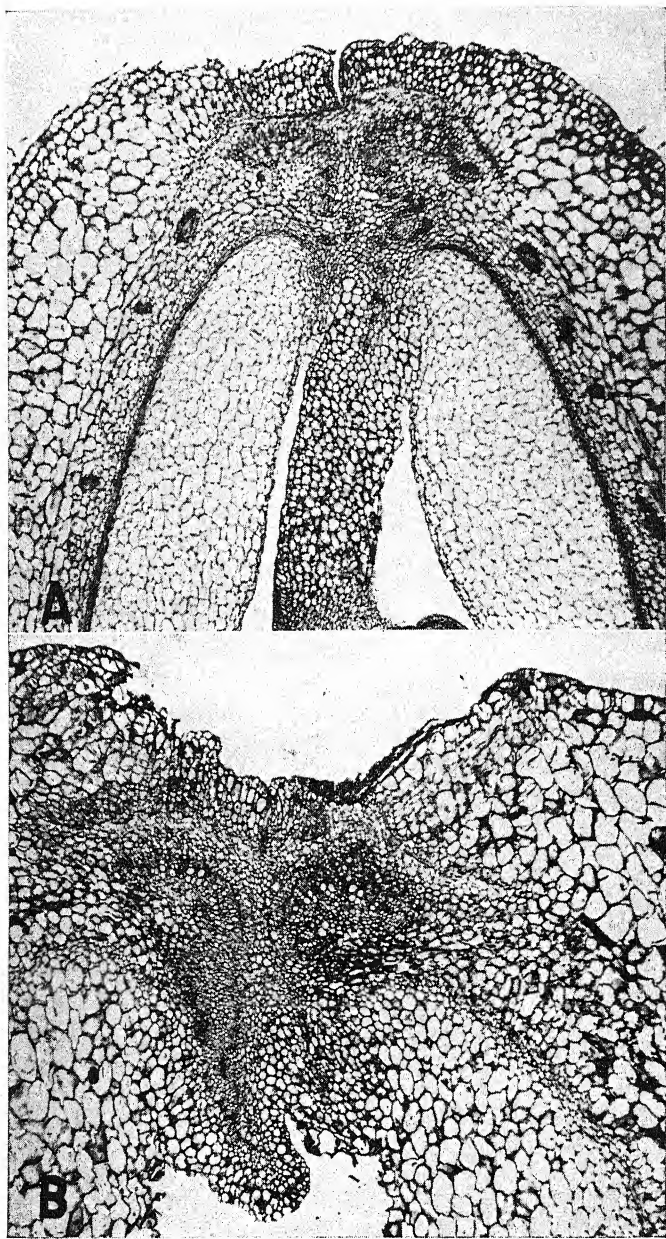


FIG. 44.—Pods treated along adaxial suture. *A*: check, abraded with emery cloth and fixed at once. *B*: 5 days after treatment with 3 % indoleacetic acid lanolin mixture. Cells of exocarp markedly meristematic; other tissues show no response.

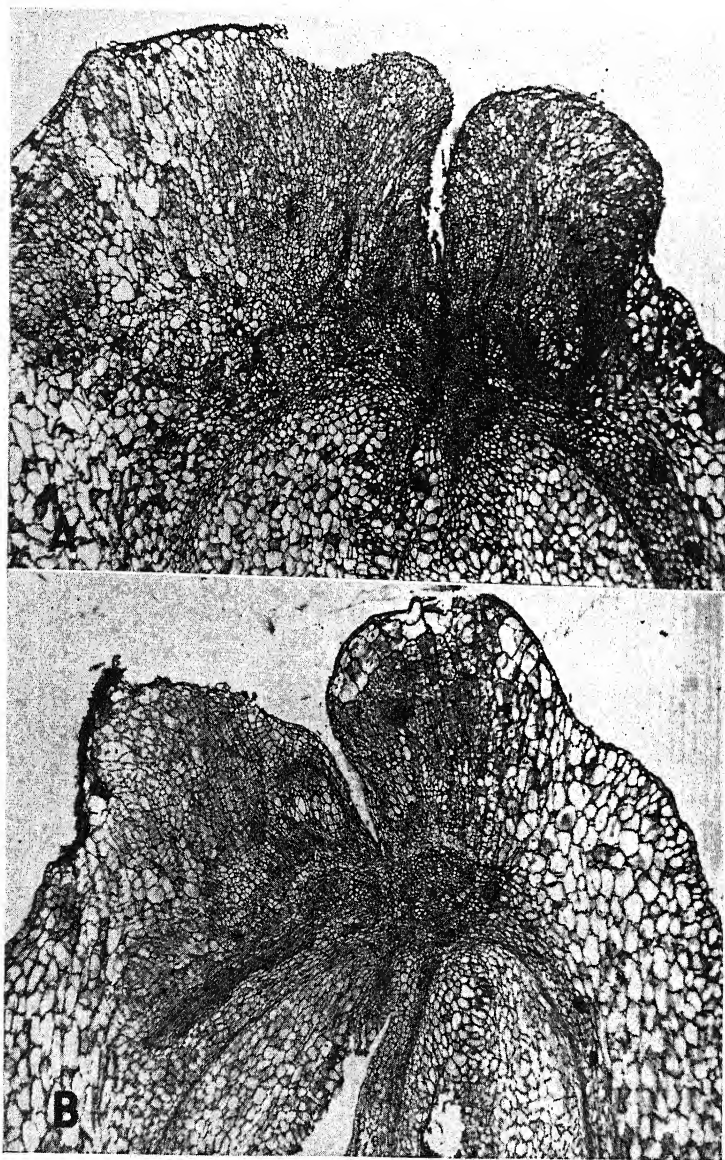


FIG. 45.—Two similar pods 6 days after treatment. Meristematic activity of exocarpic cells very great. Some derivatives are differentiating as vascular strands; others remain parenchymatous. Activity is greatest near surface but is still abundant to fibers of adaxial bundles. Mesocarpic and endocarpic cells not meristematic.

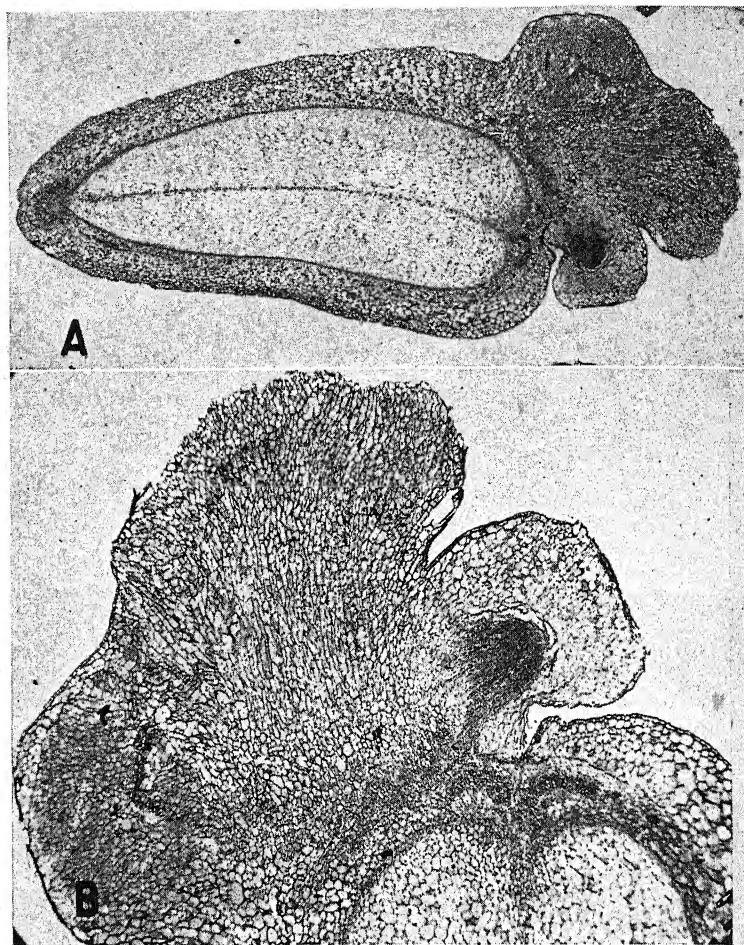


FIG. 46.—Similar pod 11 days after treatment. Proliferation almost completely limited to exocarpic tissues. In addition to vascular masses, a root has differentiated from derivatives. *B*: enlarged portion of *A*.



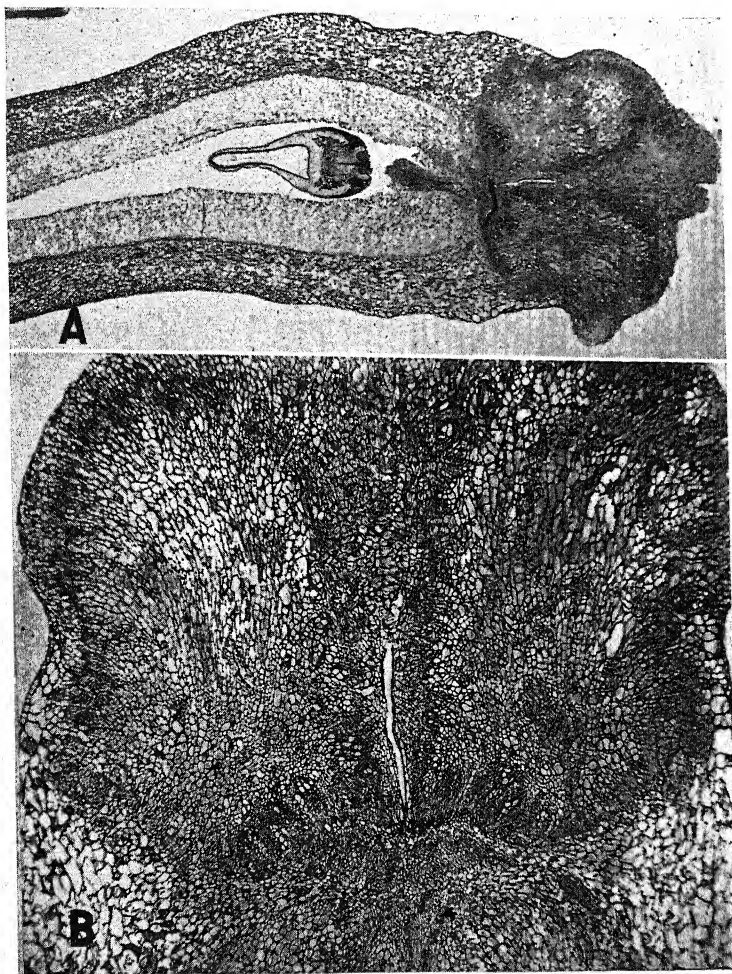


FIG. 47.—Similar pods 12 days after treatment. Proliferation almost exclusively exocarpic in origin, although there is some activity of parenchymatous cells of one of adaxial bundles just below and to left of large elongated cavity at lower center. Seed has aborted. *B*: enlarged portion of *A*.

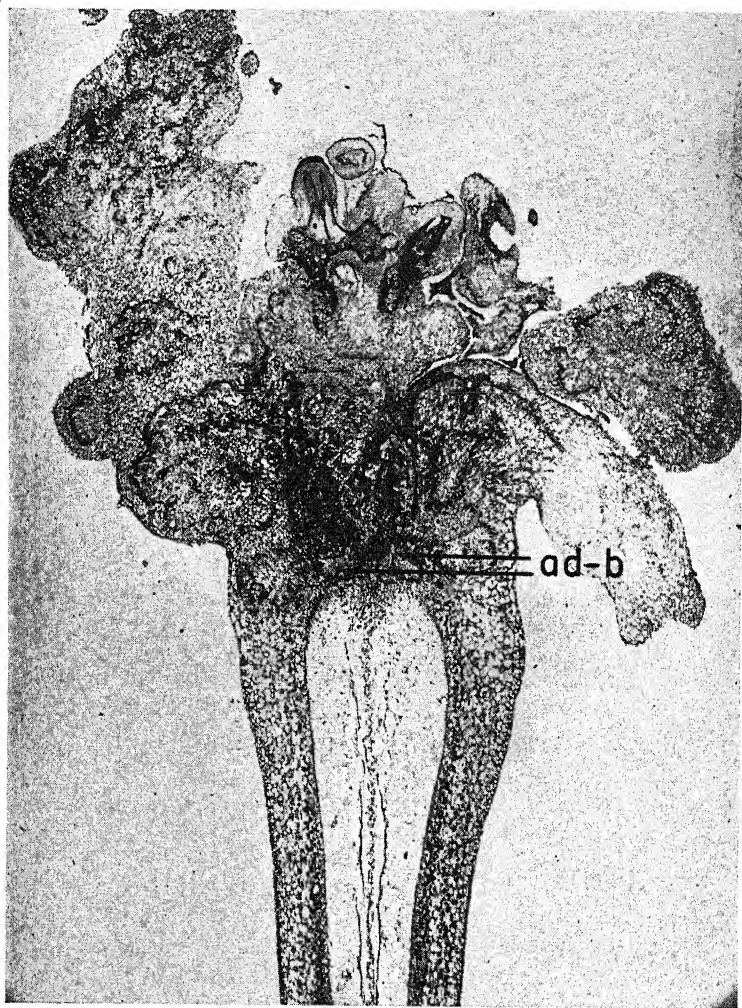


FIG. 48.—Similar pod 20 days after treatment. Practically entire tuberculated proliferation is from cells of exocarp. At upper center are several roots differentiated from same tissue. Adaxial bundles (*ad b*) show no meristematic response, nor does endocarp.

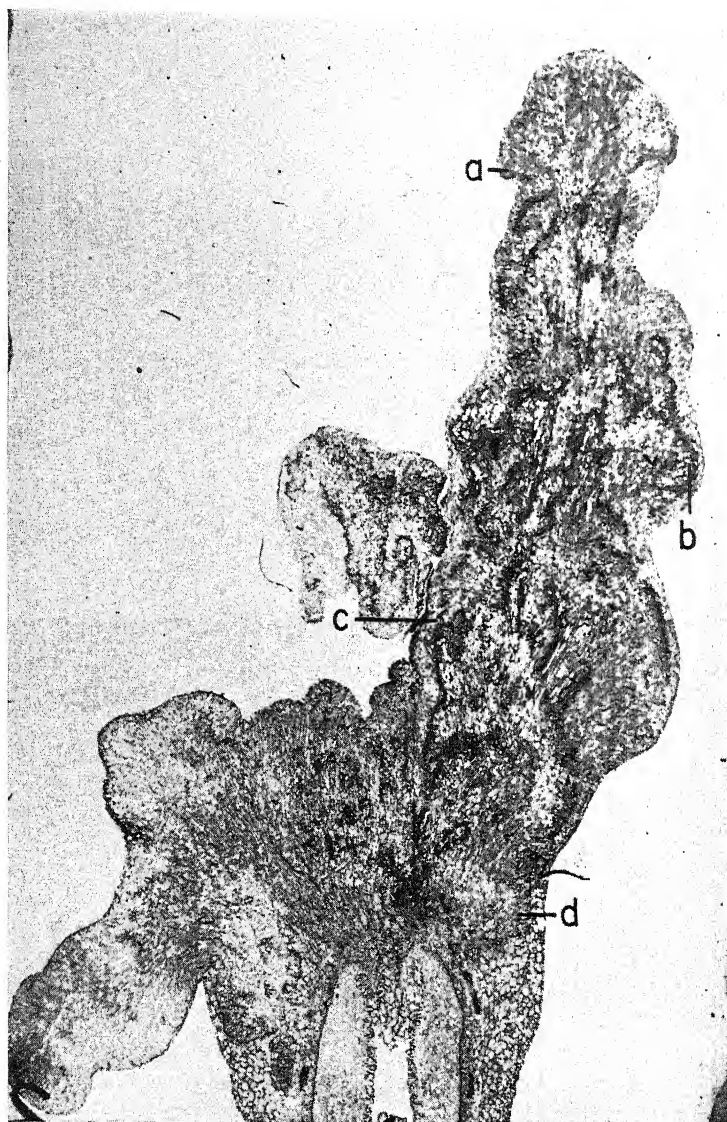


FIG. 49.—Similar pod 41 days after treatment. Most of tumor has been derived from proliferation of cells of exocarp and their subsequent differentiation, although a very small portion of it is derived from mesocarp near adaxial bundles. When collected, tumor was still actively developing, mainly peripherally, but many deep lying masses of parenchymatous tissue were highly meristematic. Letters *a-d* refer to regions shown enlarged in subsequent figures.



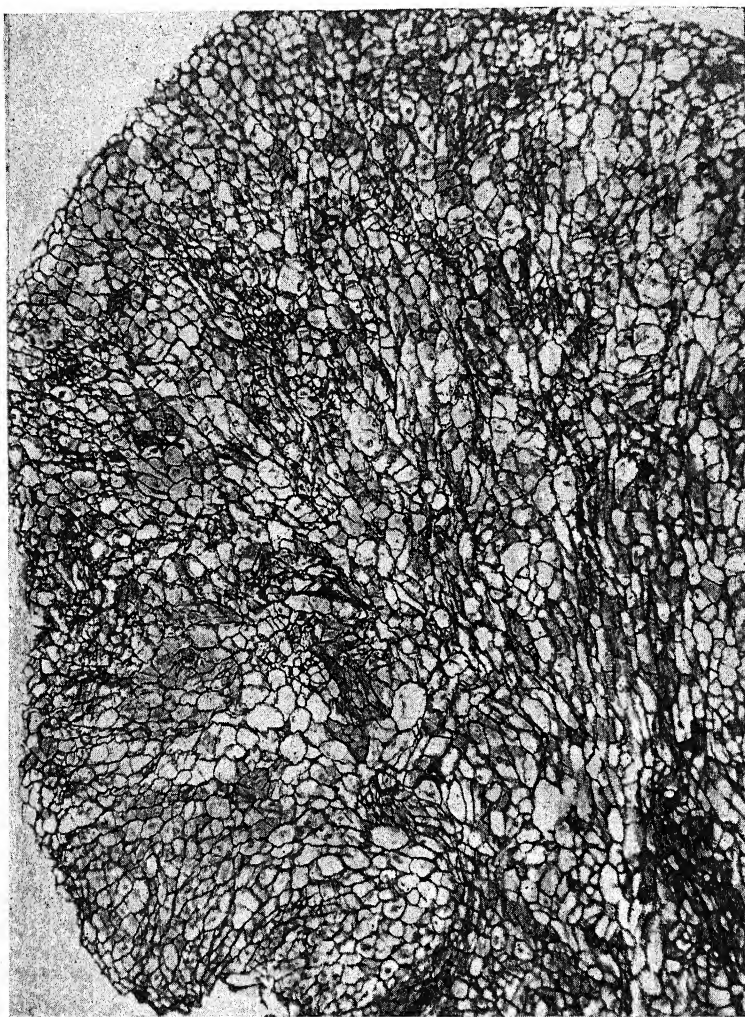


FIG. 50.—Enlarged portion of fig. 49 at *a*. Most cells of whole apical portion of tumor are actively meristematic. There are patches of parenchymatous cells which are more highly meristematic, while other groups are becoming differentiated as various types of vascular elements, sometimes as vascular strands consisting of distinct xylem and phloem portions with cambial zone between.

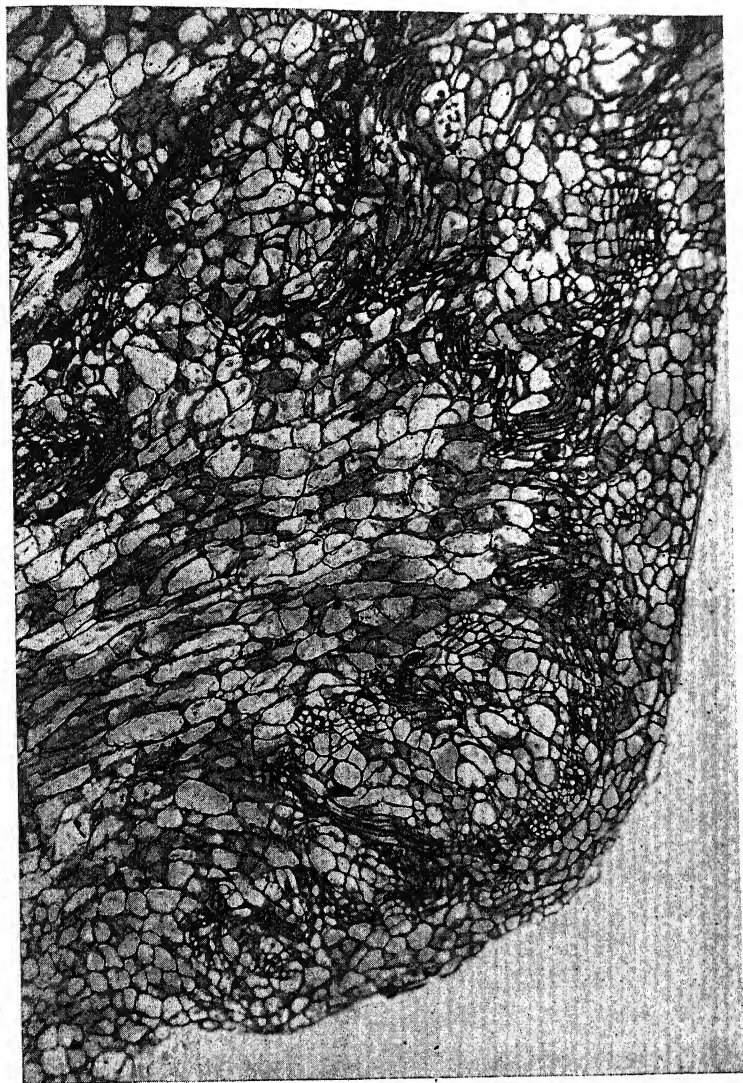


FIG. 51.—Enlarged portion of fig. 49 at *b*. A few cell layers below the surface large vascular masses have differentiated, with cambium-like zones between xylem and phloem portions. Still nearer the surfaces are patches of meristematic cells from which similar vascular masses separated by parenchymatous ones are later differentiated.

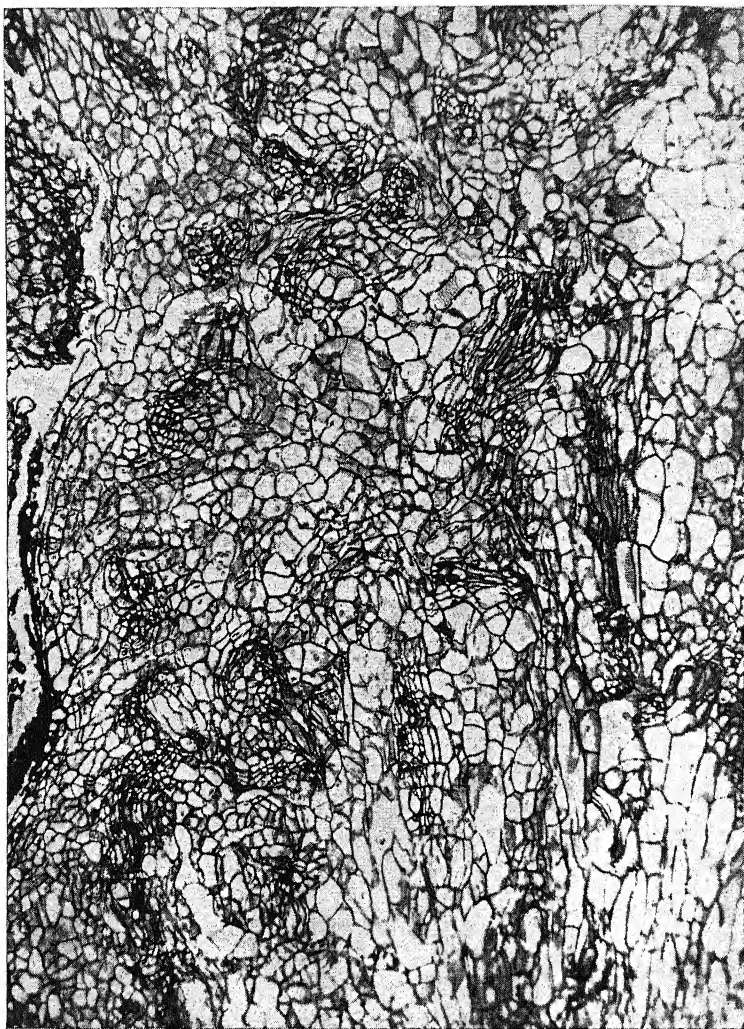


FIG. 52.—Enlarged portion of fig. 49 at *c*. This portion is similar to that at *b* except that vascular masses are smaller and more numerous, and some superficial cells which have died are being shed.

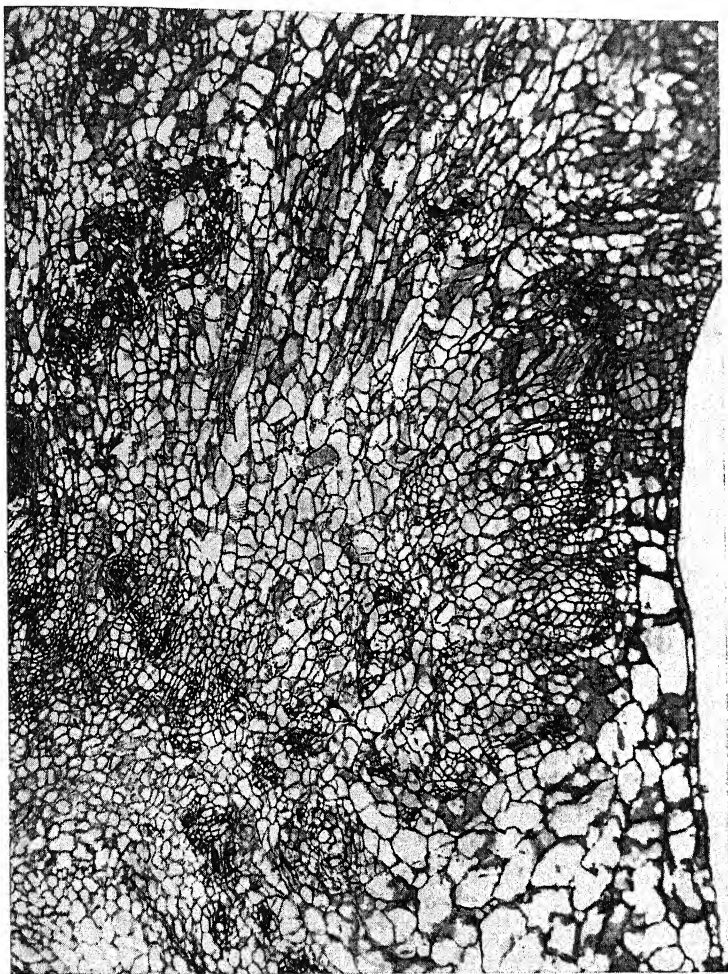


FIG. 53.—Enlarged portion of fig. 49 at *d*. In addition to same features as previously shown, at left lower corner the parenchymatous cells of one of adaxial bundles have proliferated, thus shoving some tracheids and vessels apart, and from some of the parenchymatous derivatives additional vascular strands and masses are being differentiated.

patches separated by patches of actively dividing cells. Such proliferation and maturation continued with great rapidity for many days, so that large roughened masses or tumors were formed. The more deeply lying cells of the mesocarp may not show any meristematic activity for several weeks after application of the mixture. In other instances after five to seven days the parenchymatous cells lying between the vascular bundles of the mesocarp or some of the parenchymatous cells of the adaxial bundles may become meristematic, and from such derived cells additional vascular patches, interspersed with highly active parenchymatous cells, are differentiated. Root primordia were frequently differentiated from the exocarpic derivatives (fig. 46). After several weeks of development, a section through a tumor (fig. 49) shows that most of its bulk has been derived from continued proliferation of the derivatives of the cells which composed the exocarp, although some of the parenchymatous cells of the mesocarp may also have proliferated and differentiated. Immediately under the surface there are bands and patches of highly meristematic cells and just underneath these are enlarged parenchymatous cells, patches, and strands of vascular elements in every possible degree of differentiation, as well as patches of actively dividing parenchyma. Thus activity continues indefinitely throughout the tumor, although this is most intense near the surface and is especially marked just beneath small bits of the mixture which remain on the surface of the tumor and are carried outward by the differentiation of tissues beneath them.

### Discussion

The results thus far obtained indicate that any parenchymatous tissues of the epicotyl may become meristematic when indoleacetic acid is applied to them, and of these the derivatives may differentiate into vascular structures, with the exception of the epidermis (including under this term the endocarp of the pod as here delimited) and the possible exception of the cells of the pericycle. Considerable further study with reference to the latter is necessary.

It is obvious that the same general types of responses follow the application of indoleacetic, indolebutyric, or naphthaleneacetic acid, although there is an appreciable difference in the degree or intensity

of response of the several tissues in relation to these specific substances. There is more difference in response with respect to the kind of substance employed than to the concentration of a single substance when it is applied over a rather wide range of the higher concentrations in a mixture with lanolin. It is, of course, difficult to say exactly just what may be the concentration of the substance which reaches any cell or group of cells when applications are made in this manner. The substance no doubt is soluble and diffuses from the lanolin into and among the cells of the plant, but the rate at which this occurs is difficult to determine. It is very doubtful, however, whether the increased rate of root growth which takes place when the tumors are surrounded by a saturated atmosphere, or some other wet medium, is brought about by an increased concentration of growth substance acting immediately upon roots, for when stronger concentrations are applied directly either in a relatively dry or a saturated atmosphere the roots are frequently killed rather than stimulated to greater development.

The experiments dealing with the lateral application of the mixture are suggestive in relation to the course of travel of the indoleacetic acid through the plant. It will be recalled that when the stems are decapitated and the mixture applied to the cut surface there is marked stimulation and proliferation of the pith cells adjacent to the primary xylem vessels within 48 hours for some distance down the stem, and that this proliferation continues and increases as time elapses until all the pith cells become involved. Apparently some of the indoleacetic acid may travel in vessels of the xylem. In the case of the lateral applications, however, the pith cells rarely show any activity whatsoever at any time, and even the ray cells adjacent to the xylem proliferate but little or not at all, except when the plants are grown continuously in darkness. The outer ray cells lateral to the phloem do proliferate and produce roots very abundantly. The cambium and most of the parenchymatous tissues exterior to it proliferate much as they do when the mixture is applied terminally, but centripetal to this region there is practically no proliferation. Once proliferation has begun much of the continued division and differentiation of cells is peripheral. But it is by no means all peripheral, for even in the largest and oldest apical



tumors, and in the lateral ones also, patches of highly meristematic cells are found deeply imbedded among the vascular masses. There is little doubt, however, that a continued diffusion of the indoleacetic acid to the cells is essential, as is indicated by the more rapid building up of tubercles when there is a bit of the mixture at its tip, even though the amount may be very small. If this material is removed any particular tubercle may cease development completely, although in many instances new tubercles will start into active growth in the depression between the larger ones; and so far as can be detected there is no mixture at their tips. Diffusion through the meristematic cells must take place if proliferation of cells is used as a criterion for the presence of indoleacetic acid, because such patches of cells are relatively large and at considerable distance from any vascular element. Conduction through tracheids or other xylem or phloem elements which are differentiated throughout and among these meristematic islands may transport it more readily.

Why the rhythmic development occurs both in the apical tumors and particularly strikingly so in the lateral tumors is yet without clear explanation, as are many other occurrences, such as the very rapid early development of the roots, then their almost complete cessation of growth unless the atmosphere is very moist. If the atmosphere is nearly saturated, development is almost continuous from the time of application of the mixture, and the number produced is greatly augmented. Whether in differentiation the formation of many or few roots is to take place, or whether large numbers of vascular strands are to be differentiated from the endodermal and phloic derivatives, or whether there is to be a continued proliferation and the building up of large masses of vascular tissues as tuberculate masses may well depend upon specific environmental conditions which have escaped detection or delimitation because of the relatively generalized rather than the precisely controlled conditions under which the plants were grown. Certain it is that the generalized patterns are similar under a wider range of environment, but it is equally certain that the degree of expression of any particular response varies under different environments, such as variation in moisture, temperature, darkness, exposure to light, variation in

light quality and intensity, nutrients, and other factors. All these remain for exact testing.

The experiments on the pods with the 3 per cent indoleacetic lanolin mixture indicate that the general types of responses are similar to those of stems. As already suggested, the response of the endocarp closely parallels the response of the epidermis so far observed, whether on pod, stem, or leaf, except that its meristematic activity is greater. The responses of the mesocarp are similar to those of the vascular bundles and rays between them, and those of the exocarp resemble those of the cortical tissues. While specific experiments have not yet been precisely conducted to test the point, the failure of the seeds to continue to develop when applications along the adaxial suture are made may be related to a diversion of the food supply from the seeds to the developing tumors. If there is but slight tumor development, seeds often do develop; conversely, the generally smaller apical tumor formed when the pods are cut and treated apically and the seeds continue to develop may well be related to a smaller amount of foods or nutrient materials which reach the cells composing the tumor.

Before it is possible to discuss the direct qualitative effect of these growth promoting substances on cells and cellular differentiation, some clearer and more definite means for exact quantitative determination of these substances, particularly in the tissues, must be found. The present method of attempting to estimate them quantitatively on the basis of the intensity or degree of reaction of specific cells or tissues is often not reliable, and is unsatisfactory because such intensity or reaction is dependent on various environmental factors as well as on the quantity of growth substance present or supplied. The variation which exists among the several individuals of any lot of plants grown from the purest strains of seeds now available, or among individuals which have been propagated vegetatively, is relatively large. Further experimental evidence, gained under precisely controlled experimental conditions, is necessary before many of the present or even future interpretations and hypotheses can have much real meaning.

The close similarity of tumors resulting from the application of



indoleacetic acid lanolin mixture and those produced by an extract from *Bacterium tumefaciens* has been pointed out previously by BROWN and GARDNER (3), and some of the histological similarities of the two types of tumors have been recorded (5). The close resemblance of the tumors resulting from application of lanolin mixtures of indoleacetic acid, indolebutyric acid, or naphthaleneacetic acid shows that many of the responses made by the Red Kidney bean are not absolutely specific or diagnostic for any one of these substances. Such additional evidence as has been gained, however, seems to strengthen the suggestions of BROWN and GARDNER.

### Summary

1. Apical tumors developed following the application of 3 per cent indoleacetic acid lanolin mixture to cut surfaces of decapitated bean plants may continue development for periods longer than six months and attain diameters of 2 cm. or more. Such tumors are developed largely from proliferations of the cells composing the pith, some of which differentiate into vascular elements variously arranged and anastomosed; others remain more or less actively meristematic.

2. Large, irregular vascular tumors are developed when similar mixture is applied as a narrow band encircling an uninjured young internode of the stem. Response is about equal above and below the band under average greenhouse conditions.

3. The stems to which such bands have been applied when grown under average greenhouse conditions are not retarded in their apical extension but grow and fruit as well as untreated stems under comparable environmental conditions. The pith of stems treated in this manner shows virtually no meristematic response, but develops as does that of stems which have been untreated. The roots developed are related to the rays in the same manner as those developed in apical tumors of decapitated plants.

4. There are more or less definite rhythmic periods of rapid development and comparative quiescence in the development of lateral tumors through an extended period. The irregular lateral outgrowths or tubercles of lateral tumors are developed mainly from derivatives of the phloem and to some extent from the cortex.

5. Apical tumors resulting from the application of 1.5 per cent indolebutyric acid in lanolin to decapitated stems were similar to those resulting from applications of 3 or 1.5 per cent indoleacetic acid mixture. They differ mainly in having somewhat fewer, thicker roots in the upper zone, more roots produced 1 cm. or so below the treated surface, and several tiers of roots developed between these two regions. There is slightly greater delay in the formation of the apical tumor above the treated surface, but eventually it develops to as large size, although its surface is generally smoother and less tuberculated than that developed following treatment with indoleacetic mixture. There is marked swelling of the stem and differentiation of vascular strands for several centimeters below the treated surface.

6. Apical tumors resulting from the application of 0.5 per cent naphthaleneacetic acid in lanolin differed from those following the application of the other two substances in having greater, more uniform diameter for a distance of about 1 cm. below the cut surface, where a circle of large root primordia is formed, each primordium over one of the main vascular bundles. Below these primordia the tumor tapers sharply to a much smaller diameter, giving it a distinct shouldered appearance. Below it prominent ridges extend for several centimeters down the stem. There is much less proliferation of the pith; only a slight tumor is developed above the surface of application.

7. Apical tumors resulting from the application of 1.5 per cent mixtures of each of the substances to individual stems showed greater differences than tumors produced following the application of weaker or stronger concentrations of the same substance.

8. Applications of 3 per cent indoleacetic mixture to the cut surfaces of partially mature pods which had their tips removed resulted in the production of very large vascular apical tumors and roots. The tissues composing such tumors were derived mainly from the proliferated exocarp and mesocarp of the pod. The endocarp which in untreated pods is principally derived from proliferated epidermal cells becomes somewhat more active in treated pods, but forms little or no part of the apical tumor; nor have vascular elements differentiated from this proliferated tissue been observed.

9. The large apical tumors are built up from derivatives of cells which originally were near to or but a relatively few cell layers away from the treated surface, a far shorter distance than is the case in stems.

10. In at least half the apically treated pods the seeds abort. If the seeds continue to develop, the apical tumor produced is generally smaller than if they abort.

11. When the epidermis at the adaxial suture of partially developed pods is slightly abraded and 3 per cent indoleacetic lanolin mixture applied, large vascular tumors are developed from the proliferated exocarp and to a far lesser degree from the mesocarp. In such pods most of the seeds abort.

12. The quantitative response made by stems or pods when treated with any one of the three substances used in mixture with lanolin was influenced more pronouncedly by small variations in the environment or the state of vigor or development of the plant than by comparatively great variations in the concentration of the particular mixture used or in the amount of the mixture applied. There was less difference in this respect in the qualitative than in the quantitative response.

13. Caution is required, therefore, in any endeavor to make a quantitative estimate of the amount of any of these substances which may be present in the living tissues of the plant if such estimate is based on a quantitative response of tissues in close proximity to the place of application or distant from it.

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## MORPHOLOGY OF CUPRESSUS ARIZONICA: GAMETOPHYTES AND EMBRYOGENY

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(WITH FOURTEEN FIGURES)

### Literature and object

BUCHHOLZ (1-5) has made wide use of comparative embryogeny to determine nearness of kinship among conifers. The extensiveness of his work was made possible by the perfection of a dissection technique (4) which permits the examination of embryos and coiled suspensor systems without the tedium of sectioning, and without the difficulty of subsequent interpretation from fragments which sectioning of coiled structures entails. He has made extensive use of the stages between the organization of the proembryo and the organization of the tissue regions of the embryo. Both in the works of BUCHHOLZ and in the recent summary of conifer embryogeny by SCHNARF (8), comparative embryogeny is found to be of taxonomic value. This emphasizes the necessity of our acquiring a wide knowledge of embryogeny before we can consider complete the work arranging the constituents of the Coniferales into a natural system.

Information regarding representatives from most of the genera is now available, but our knowledge of the embryogeny of genera from the tribe Cupressineae is distinctly limited. *Chamaecyparis* (5), *Thuja* (7), *Biota* (4), and only a few others have been investigated. Despite the uniformity of embryo type which usually exists among the members of a closely related group, two markedly different types have appeared among the investigated members of this tribe. It becomes of interest, therefore, to know into which of these groups uninvestigated members will fall or whether additional types will be discovered. These questions as they relate to *Cupressus arizonica* are given consideration in the present paper.

The morphology of the male gametophytes of this species was reported in an earlier work (6). Most of the material for the present study was taken from the same tree which furnished the material

for the previous investigation. Although *C. arizonica* is indigenous to the mountainous portions of Texas, it is not native in the region adjacent to College Station. The present findings, therefore, were checked against material obtained from trees grown at Alpine, Texas, where conditions closely approximate those under which *C. arizonica* grows naturally.

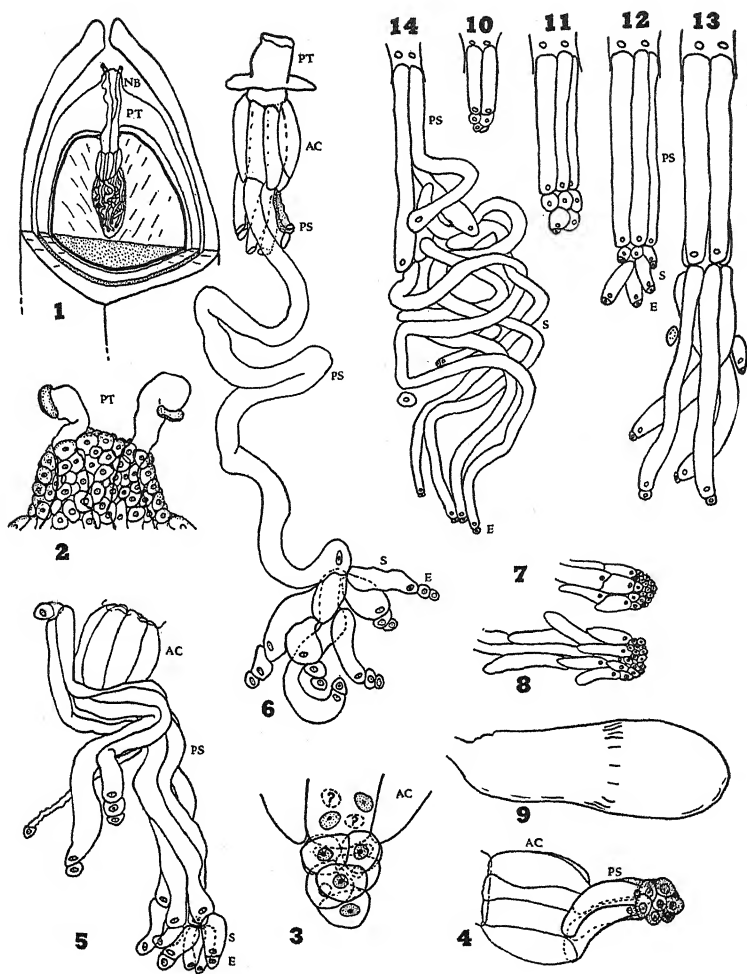
### Material and methods

At College Station some plants produce both pollen cones and seed cones regularly, and still others none at all. In some trees there is a decided tendency toward monoecism, especially in young specimens of the second generation under domestication. Plants vary widely in the time at which the pollen is brought to maturity. Ovules with copious pollination droplets have been seen as early as January 30 and as late as March 15. Late spring frosts often kill some or all of the pollen cones. The plant from which most of the material was taken, however, has been a consistent producer of viable seed and has many offspring in this region. Seedlings vary widely in form and color, indicating considerable heterozygosity.

Parallel embryo studies were made in the summers of 1929, 1932, and 1936. Checks have been made against material from other trees in this region and from trees at Alpine. No significant differences were found from tree to tree or from season to season.

The ovules were opened with a razor blade. The nucelli with their inclosed female gametophytes were removed and dropped into 0.3 gm. molecular cane sugar. In this, as well as in the dissecting, staining, and mounting, the technique of BUCHHOLZ was followed. It was found possible to make camera lucida tracings of embryos freshly dissected and thus to obviate the necessity of mounting all the embryos.

*Cupressus arizonica* has at the apex of the nucellus a more or less prominent "nucellar beak" (figs. 1, 2) upon which the pollen grains germinate. The resulting tubes later lift the empty exines away from the surface of the beak, where they remain conspicuously elevated (figs. 1, 2). As a matter of routine, the nucellar beaks were examined for the number of their pollen tubes before the female gametophytes were removed from the nucelli.



FIGS. 1-14.—Fig. 1, semidiagrammatic drawing of sectioned upper portion of developing seed in midsummer of the first year. Fig. 2, detailed view of nucellar beak with protruding pollen tubes. Fig. 3, late proembryo; upper nuclei were overlain by archegonia and their number could not be definitely determined. Fig. 4, slightly older stage showing single embryo system with elongated prosuspensors. Fig. 5, still older stage showing two embryo systems; one of the units in that on the left did not elongate and one prosuspensor is undergoing collapse; embryo system on right is more nearly typical. Fig. 6, still older stage showing archegonial complex with two embryo systems from which seven of the eight prosuspensor units, together with their derivatives, have been omitted; prosuspensor elongation is complete and suspensors are beginning elongation. Figs. 7-9, functional (apical) embryos in winter rest condition; embryo shown in fig. 8 is undergoing vegetative budding. Figs. 10-14, stages in embryogeny of *Biota orientalis* as drawn by BUCHHOLZ and repeated here by permission. The steps are essentially the same as those found in *C. arizonica*. Figs. 13 and 14 represent stages beyond that shown in fig. 6. *ac*, archegonial complex; *e*, embryo; *nb*, nucellar "beak"; *ps*, prosuspensor; *pt*, pollen tube; *s*, suspensor.

It was found possible also to cut away the sides of the nucellus and to observe directly the number of tubes which had entered the archegonial chambers (figs. 1, 6).

In some cases the nucellar tissues which surround the upper reaches of the pollen tubes were dissected away and the irregular tubes exposed. An additional observation involved the counting of archegonia per archegonial complex.

### Observations

As previously reported (6), *C. arizonica* develops erect urn-shaped ovules. Within these the nucellus is free from the integument, except at the base. It is protected except for the nucellar beak by a smooth tube-proof epidermis. The beak upon which the pollen germinates lies directly beneath a rather wide and shallow micropyle.

The pollen tube follows a tortuous course and often develops numerous male cells, which may fertilize numerous eggs within the same archegonial complex. Fertilization is accomplished about June 1, and two seasons are required for the ripening of the seed.

More than half the nucellar beaks examined received no pollen. Some pollen failed to germinate. A sample distribution of 300 pollen grains is summarized in table 1.

TABLE 1

GERMINATED GRAINS ON NUCELLAR BEAK					TUBES IN ARCHEGONIAL CHAMBER				TUBES NOT ARRIVED
Numbers.....	1	2	3	4	1	2	3	4	
Cases.....	262	34	3	1	196	7	1(?)	0	96

No case was found in which more than four grains had germinated on a single nucellar beak, and only once were three pollen tubes found entering a single archegonial cavity.

Some of the examinations were made before the tubes began to arrive at the archegonial chamber or before arrival was completed (some had not arrived in mid-July). For this reason a comparison of the number of tubes starting growth and finishing at the archegonial chamber has little significance.



Tubes in the same nucellus may arrive simultaneously or in succession. Some may not arrive at all.

The observed numbers of archegonia per complex ranged from one to thirteen. A single archegonium was observed but once, and its unusual size indicated abnormality. A sample distribution of archegonial numbers for 100 complexes counted at random is furnished in table 2.

Since a paper on multiple male cells (6) covered stages up to and including fertilization, a description of the proembryo logically falls next in order. The dissection technique, however, proved unsuited to the study of nuclear divisions and wall formation in the early proembryo. When dissected in the early stages of zygotic division,

TABLE 2  
DISTRIBUTION OF NUMBER PER ARCHEGONIAL COMPLEX  
FOR 100 FEMALE GAMETOPHYTES

No. of archegonia.....3	4	5	6	7	8	9	10	11	12	13	Total
Frequency.....4	3	9	11	29	19	12	7	3	2	1	100

overlying archegonia and attached jacket cells obscure the early stages of the developing proembryo. In its later stages, however, the cells of its protruding tip may be seen below the apices of the archegonia. The elongation of certain cells to form a prosuspensor begins before the proembryo has reached the typical 16-celled stage so characteristic of pines and cedars. The even tiers of four cells are conspicuously absent (fig. 3). The early transformation of the cells homologous to the rosette tier of the pine proembryo into an elongated prosuspensor deprives the proembryo of *Cupressus* of its potential rosette layer, and in effect adds the cells of this layer to the suspensor system (figs. 4, 5). No rosette embryos were observed. Occasionally, however, a potential prosuspensor failed to make the usual elongation, and its attached embryo when crowded back gave the appearance of having arisen from the rosette (upper left, fig. 5).

In early stages the tubular units of the prosuspensor can be counted, and at this time their number is of value in determining the number of embryo systems (embryos from one zygote) in the entire

embryo complex (embryos from all zygotes). There is roughly one embryo system and of course one zygote for each group of four prosuspensor tubes (figs. 4, 5, 10-14). In my material the prosuspensors ranged in number from four to twenty, indicating from one to five zygotes. An embryo complex which shows twenty prosuspensor tubes cannot have resulted from less than five zygotes, and yet such a complex was observed in a female gametophyte to which there was attached but a single pollen tube. Numerous cases of three or more zygotes from a single tube were observed. Apparently either the multiple male cells from a single pollen tube effect multiple fertilizations or some of the embryos arise parthenogenetically. Since this species has been shown to have multiple male cells, the multiple fertilizations are more likely the correct explanation.

The elongation of the prosuspensor may or may not be followed immediately by the elongation of the true suspensor cells (figs. 5, 6).

Often the points of contact between the prosuspensor tubes and suspensor tubes are slight. In approximately 10 per cent of the cases they become detached and subsequently tangled beyond all hope of separation into distinct embryo systems.

In many cases vertical wall formation begins immediately following elongation of the prosuspensor, thus giving rise to a large number of loosely connected embryos with varying numbers of short suspensors and embryonal tubes (fig. 6). They may become completely separated from their connections, either by collapse and subsequent digestion of the connecting tubes, or by simple detachment of abutting tubes along their contiguous ends. In rare cases balloon-like distensions are formed.

A far more typical behavior is for the vertical wall formations, separations, and distensions just described to be postponed until after the elongation of the true suspensor cells (figs. 13, 14). At about this stage, which is reached approximately July 1, some of the single celled embryos temporarily cease dividing and go into a semi-thick walled condition similar to but less pronounced than that reported for *Biota* (4).

For most of the embryos development continues slowly through July, August, and a part of September, by which time all embryos

have been eliminated except the functional one. Of the dozen to five dozen embryos which may have arisen in a complex in the course of the summer, usually only four or five remain at the onset of the winter rest. Among these the functional one is usually clearly recognizable. This, the largest of the group, is crowded into the apex of the embryo cavity and there remains inactive during the winter period. At this time the embryos vary widely in their size and degree of development. Some show unmistakable sign of vegetative budding (fig. 8). Prominent embryonal tubes anchor the embryos firmly in place (fig. 8) among the partly digested cells of the female gametophyte. Although the embryos are, during the winter rest, rarely if ever in possession of tissue regions and cotyledonary primordia, they initiate these immediately upon resumption of active growth (figs. 7-9), and by the middle of April most of the embryos have reached full size.

It is clear that in all particulars, save the winter rest period, the development of the embryo of *C. arizonica* follows, to a remarkable degree, that already described by BUCHHOLZ for *Chamaecyparis obtusa* (5) and for *Biota* (4). In order to emphasize this fact, figures 10 to 14, which are copies of BUCHHOLZ's diagram of the embryogeny of *Biota*, are furnished herewith. They could as well have been drawn for *Cupressus*.

*Biota* is sometimes merged with *Thuja*. The former, as already pointed out, has extreme cleavage polyembryony. The latter has simple polyembryony and no cleavage. The evidence furnished by this report, therefore, can be considered to support the contention that the genus *Biota* of ENDLICHER is valid, and that from the evidence of its embryogeny, it lies closer to *Cupressus* and *Chamaecyparis* than to *Thuja*.

### Summary

1. The steps in the early embryogeny of *Cupressus arizonica* are described.
2. The number of archegonia in a complex is found to range between 3 and 13.

3. More embryo systems were observed than could be accounted for on a basis of two fertilizations per pollen tube, indicating multiple fertilizations from multiple male cells.

4. The steps in the embryogeny, except for the speed of their passage and the insertion of a winter rest period, closely parallel those of *Biota orientalis*.

5. The resemblance of the embryogeny of *C. arizonica* to that of *Biota* is taken as further evidence that ENDLICHER's genus *Biota* is valid, and that it more closely resembles *Cupressus* than *Thuja*, with which it is sometimes combined.

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RESPONSES OF BEAN AND TOMATO TO PHYTOMONAS  
TUMEFACIENS, P. TUMEFACIENS EXTRACTS  
 $\beta$ -INDOLEACETIC ACID, AND WOUNDING<sup>1</sup>

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 480

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(WITH TWENTY-TWO FIGURES)

Introduction

In our study of nutrition of tomato relative to crown gall development (23), we corroborated (24) BROWN and GARDNER's findings (5) that *P. tumefaciens* (Smith and Town.) BERGEY *et al.*, incitant of crown gall, produces growth substances. Further, the crude extract was found to contain heteroauxin (24). To date scarcity of material has prevented analysis for other significant constituents. Application of the extracts and of heteroauxin was made with and without wounding to the bean and tomato. Wound responses of the bean in control experiments led to extended study of its reaction to puncture, incision, decapitation, and segmentation.

Because of recent excellent summaries on plant hormones and their effects, there is no need for a literature review in this paper. AVERY and BURKHOLDER's translation and revision (3) of BOYSEN-JENSEN's monograph and the second review by JOST (13) cite original and review papers. There is relatively little pertinent literature on *Phaseolus vulgaris*. European workers almost invariably use *P. multiflorus* (4, 10). WEHNELT (33) does not state what species of *Phaseolus* he used. SILBERSCHMIDT and KRAMER (30) used *P. vulgaris*. KÜSTER's text cites the earlier literature on pathic tissues of this plant (17). BROWN and GARDNER (5), KRAUS, BROWN, and HAMNER (16), and HAMNER and KRAUS (9) have used *P. vulgaris*.

The voluminous literature on crown gall has been reviewed by RIKER and BERGE (28). Hence no attempt is made to treat it here.

<sup>1</sup> Supported in part by a grant from the Rockefeller Foundation to the University of Chicago. Additional cost of publication sustained by the writers.

### Materials and methods

Experiments were performed from May 1936 through the following spring. The bean rather than the tomato was selected as chief test plant, since it lends itself more readily to greenhouse culture during Chicago winter conditions, develops rapidly with a massive seedling and later with large well defined members, and is less disposed to develop aerial roots under normal greenhouse conditions. The bean also produces nodules. The only troublesome infectious diseases were mildew, controlled by sulphuring, and blight, controlled by use of infection-free seed. *Phaseolus vulgaris* var. Red Kidney, although not quite so responsive as Golden Wax, was used because BROWN and GARDNER (5) and KRAUS and associates (9, 16) have used it. Tomato varieties Marglobe, Bonnie Best, and Ponderosa were used. No differences were noted in their behavior.

Day and night winter temperatures were approximately 70°-75° F. and 60° F. respectively. From March on, day temperatures tended to be higher (90° F.). Humidity in the houses was relatively low, Wardian chambers being used when high humidities were desired. Most plants were grown in soil under these conditions. The form of the plants changed as the season advanced, hypocotyl and internodes becoming shorter with increasing daylight. In addition beans were grown in the dark in quartz sand or soil at 80° F. and a relative humidity of 70 per cent. Beans grown in quartz sand in the light for prolonged periods were not so desirable for experimental purposes because of deficient growth. Some tomatoes grown in quartz sand received plus or minus nitrate nutrient solutions.

Since control plants wounded by puncture, incision, and transection made some responses similar to those obtained with extract, heteroauxin paste, or *P. tumefaciens*, experiments were made, primarily with bean, on the effects of wounding. Bacterial extracts on very young bean and apical tomato tissues caused killing. Second internodes of the bean were scraped and treated with 25 per cent acetic acid to determine whether later growth responses to the extracts were wound responses following local killing. Seedlings and young plants were punctured, incised, and/or segmented with or without removal of cotyledons (fig. 3). When severed, the portions were placed on moist sand flats in the dark. Observations were

made to ten days. Punctures were made with a needle to the center of the axis in young arched hypocotyls, and in older plants in upper and lower halves of the hypocotyl, first and second internodes. Vertical and transverse incisions were made in the same locations in the older plants, some of the latter with inserted glass or mica plates. Eight days old seedlings were incised transversely in the middle of the hypocotyl or decapitated directly below the cotyledons. Severed seedlings were similarly incised, also segmented at the middle of the first internode or at top or middle of the hypocotyls. Hypocotyls were left in one piece, halved, quartered, or further cut to minimum lengths of 1 cm.

The  $\beta$ -indoleacetic acid (heteroauxin) used was obtained in crystalline form from Merck. Preliminary experiments were made with 0.2 or 2 per cent heteroauxin in lanolin applied to tomato basal and apical internodes, some of the latter being decapitated. Later experiments were made using 3 per cent heteroauxin in lanolin, following BROWN and GARDNER (5) and KRAUS and associates (9, 16). Applications were made as before to tomato with and without puncture. Bean hypocotyls of different ages were inoculated at several sites with dosages estimated at 0.01 and 0.0005 cc., applied with a tuberculin syringe. Later, following LAIBACH's method (20), concentrations were prepared ranging from 0.1165 to 0.0000122 per cent or  $1:10^3$  to  $1:10^7$ , the initial concentration being LAIBACH's 1:1 paste. Dilutions were made by weight in geometrical progression using lanolin water paste (1 part lanolin to 1 part water) as dilutant. In later experiments the 3 per cent lanolin paste was used as an additional concentration. Applications were made with and without wounding to various organs of the bean and less extensively to the tomato, unilaterally, by banding, or by covering decapitated organs. These tests were to determine the extent to which heteroauxin could induce the gamut of known pathic growth responses. With one exception, concentrations high enough to produce killing were not used. By decapitating epicotyls, blades of first leaves, and removing hypocotyls with and without applications of paste, experiments were made to determine whether foreign growth substance (heteroauxone, see discussion) can substitute for native growth substances (autoauxones, see discussion) of the bean or whether their augmenta-

tion, as in application of heteroauxin to the bean, would increase growth rate.

A strain of *P. tumefaciens* derived from a single cell isolation (W. M. Banfield) was used for direct inoculation and for preparation of all bacterial extracts. A 24 hours old culture grown on standard potato dextrose agar was applied with and without puncture to the upper and lower halves of the hypocotyl and the first and second internodes of the bean, apical and basal internodes of the tomato, and decapitated top internodes of both. An older culture grown in quantity in Kolle flasks was divided, one portion being extracted with ether, the residue taken up and applied, and the other killed with 1:10,000 merthiolate. Agar on which these organisms were cultured was applied to decapitated bean hypocotyls. The two broth media described later with 2 per cent agar added were similarly inoculated and used as a source of agar blocks in this experiment.

Galls incited by *P. tumefaciens* on tomato and control stem and leaf samples were extracted with acidulated chloroform according to THIMANN'S method (32). The residues were applied directly to the bean hypocotyl with and without puncture, and to second internodes. Gall extract only was applied to tomato seedlings, and all three to older tomatoes approximately 20 cm. tall.

In preparation of bacterial extracts, *P. tumefaciens* was cultured at 70° F. in two broth media, one containing 2 per cent dextrose and 0.1 per cent tryptophane in distilled water, the other containing in addition 1 per cent peptone. Heavy pellicles were produced in the latter. Ether extracts were made of cultures three weeks and three months old, the pellicles being extracted separately. The broth culture was shaken for several hours with an equal volume of anhydrous peroxide-free ether, then stored several days at 30° F., the ether decanted and evaporated. Pellicles, after removal from the medium by filtration through glass wool, were ground with it, and similarly treated with ether. All these preparations yielded viscous, noxious smelling, dark orange-brown residues which were stored at 50° F.

The extracts were applied directly (without mixture with lanolin) to the bean hypocotyl, first and second internodes with and without puncture, and to decapitated second internodes. First inter-



nodes of tomato seedlings and apical internodes of older plants were also treated. Applications were made to a limited number of *Avena* coleoptiles. Since the yield was very meager, a small blade hammered from a pin point was used to scrape the material from the evaporating dish and for its application to the plant. Minimum effective dosages were estimated at 0.0025 cc.

Preliminary identification of the presence of heteroauxin in the extracts was made by the hydrochloric acid, ferric chloride, amyl alcohol test specific for that substance (1, 15). The standard consisted of a saturated (0.233 per cent) aqueous solution of  $\beta$ -indoleacetic acid (Merck) diluted in geometric series to give a final concentration of 1:10<sup>4</sup>. Volumes of 20 per cent hydrochloric acid and of 0.1 per cent ferric chloride equal to that of the test material were added (0.32 cc.), and gently boiled until the cherry red color of the lower dilutions or pink color characteristic of higher dilutions of heteroauxin was obtained. Amyl alcohol, one half (0.16 cc.) the volume of any other single ingredient, was added. With brisk shaking, the color passed into the alcohol.

Five mg. lots of extract were dissolved in ether, yielding a yellowish brown solution. After addition of the acid, the ether was driven off over a water bath and the test repeated as described, using the same volumes. In the course of boiling, the characteristic red or pink color appeared and later dissolved into the alcohol, this color becoming partially obscured by the yellow color of the initial solution.<sup>2</sup>

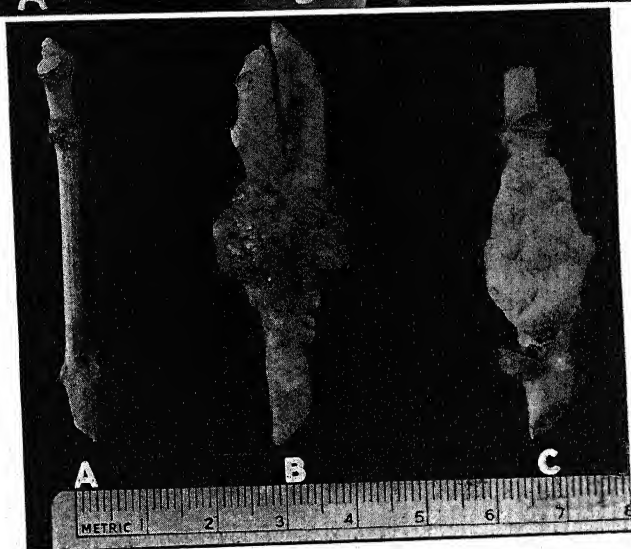
All samples for histological examination were killed and fixed in Navashin's solution.

### Experiments and results

#### INOCULATIONS WITH *P. TUMEFACIENS*

Applied without wounding, *P. tumefaciens* produces no visible effects on the tomato and bean. Introduced by puncture or decapitation in the tomato, it causes galls, abnormally abundant adventitious roots, epinasty, stunting, and death (25) (fig. 1). In the bean, puncture of the axis leads to gall formation (figs. 2A, 14G, I).

<sup>2</sup> We are indebted to Professor F. C. Кочн of the Department of Biochemistry for consultation and verification of results.



FIGS. 1, 2.—Fig. 1, A, B, moderately dry air; C, high humidity. A, control; B and C inoculated at  $x$  with *P. tumefaciens*. Note epinasty, gall, and adventitious roots in B and C. Tomato treated with heteroauxin or with extracts of *P. tumefaciens* produces identical gross effects (fig. 18). Fig. 2, A, bean; B, tomato; C, *Bryophyllum* internodes inoculated same date with *P. tumefaciens*. Galls of A and B have ceased growth; C still very active. All 6 weeks old, in moderately dry air.

Because of relatively low humidity of the room in which the plants were grown, inoculated decapitated stems did not develop the galls recorded by BROWN and GARDNER (5). Since the bean internode elongates rapidly and ceases to increase earlier than the tomato, bean galls do not attain the relative or absolute size of tomato galls (fig. 2A, B). *Bryophyllum*, being a slow but prolonged growing plant, produces galls which are still extending along the axis when tomato and bean galls of the same age have ceased growth (fig. 2C). Eventually huge galls are produced that kill the plant. In each of these plants the greatest response was provoked in the most actively growing area. The same area when older could fail entirely to respond, as in the bean hypocotyl.

#### WOUNDING

The injured bean, including all vegetative organs and the fruit (16), is highly disposed to tumor<sup>3</sup> formation, development of adventitious roots, and root regeneration (fig. 3). No data are available for floral parts. Punctures in the young internode produced local swelling (fig. 14 F, H). No enlargement occurred in the hypocotyl. Vertical incisions resulted in the usual callus formation. Transverse incisions with or without insertion of glass or mica plates caused local swelling above the wound, accompanied occasionally by roots, often fasciated, and/or marked callus formation. Similar responses occurred on basal ends of certain segments but rarely at the apical end of any segment. Apical segments of the hypocotyl and those 1 cm. in length at any level failed to respond. Longer segments showed more roots, more callus production, and less desiccation than shorter ones. Root production was usually but not always accompanied by swelling, occasionally by callus formation. In the first and second internodes, swelling occurred on incision and severing but root production was rare. Leaves severed just below the pulvinus from the petiole developed swelling at the cut.

These results indicate a definite polarization and concentration of root inciting substances. To check this hypothesis, 3 per cent

<sup>3</sup> Tumor is here used to designate any localized swelling due to anomalous growth (*i.e.*, a neoplastic swelling). Following KÜSTER, gall is used to designate a tumor incited by a parasite that derives its nutrients from the tumor tissues (17, 18). Hyperhydric tissues that produce swelling (including intumescences and surface callus), and internal callus that leads to swelling, are instances of tumors.

heteroauxin paste was applied at the apical end or at the middle of hypocotyl segments 4 cm. long. Roots appeared directly below the application.

In severed eight days old seedlings, removal of the cotyledons resulted in marked wilting, less basal swelling, fewer roots. Those produced were less well developed and often remained visible as pro-

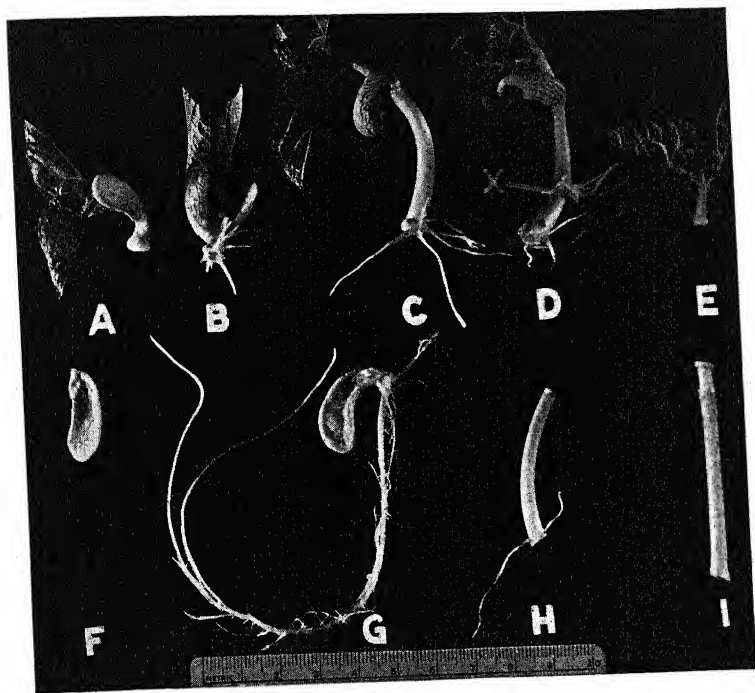


FIG. 3.—Segments of bean seedlings kept 10 days in wet sand flats in dark. *A*, severed immediately below cotyledons; swelling (internal callus) at basal end; whitening. *B*, like *A* but older; roots regenerated from swelling. Roots in all specimens in four rows, with tendency to fasciation. *C*, longer segment with swelling and longer roots. *D*, segment as *C* but incised at *x*; swelling and roots above upper surface of cut and at base. *E*, epicotyl with swelling (these occasionally develop roots). Severed leaves develop similar swellings. *F*, removed cotyledon with swelling. *G*, removed and heteroauxin treated cotyledon with large swelling and roots. *H*, segment of hypocotyl with swelling at base and top and roots at base. *I*, segment of hypocotyl and first internode decotyledonized, with swelling at base and large surface callus at top.

tuberances beneath the surface of the stem. In nine days old seedlings these differences were less marked, but of the same kind.

Development of axillary shoots following spontaneous blasting of the leaves of the embryonic epicotyl (fig. 4*D*) or experimental decapitation of the internodes (fig. 4*B, C*) indicates inhibiting effects of these and implies growth correlations dependent on polarization of the main streams of hormones moving from divergences toward and down the axis.

In the tomato, punctures gave slight swelling. Areas exposed by decapitation became covered by callus.

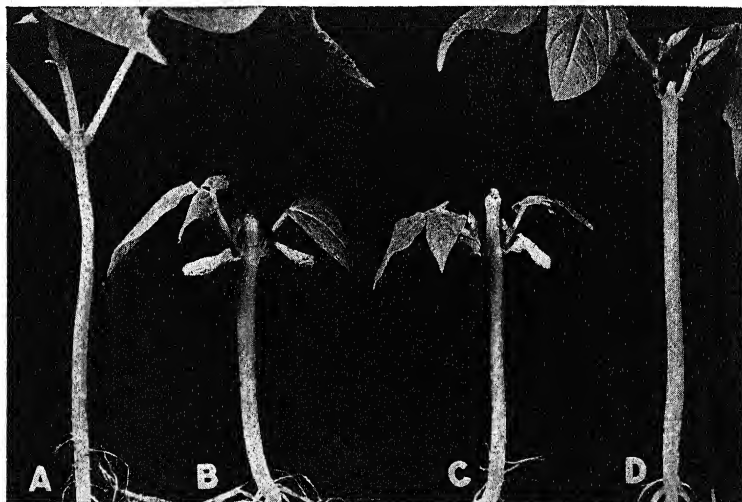


FIG. 4.—*A*, control; *B, C*, decapitated first internode. Note cotyledons still present, axillary shoots. Swelling of stump and hypocotyl due to heteroauxin paste 1:10<sup>3</sup>. *D*, seedling with spontaneous blasting of leaves of plumule. Note hypocotyl reaches second node of control, axillary cotyledonary shoots, and shriveled first internode.

#### $\beta$ -INDOLEACETIC ACID APPLICATIONS

The demonstration that *P. tumefaciens* produces heteroauxin in culture (24) led to extensive bean and limited tomato experiments with the chemically pure substance.

ROOT.—Beans were germinated in soil and in clean quartz sand. To primary roots not over 7 cm. long in the former, 3 cm. long in the latter, as small a mass of paste as possible was applied, the site being indicated by  $\alpha$  in the figures. It was found that the first 3 mm. of the root must be treated to get effects. The seedlings were then

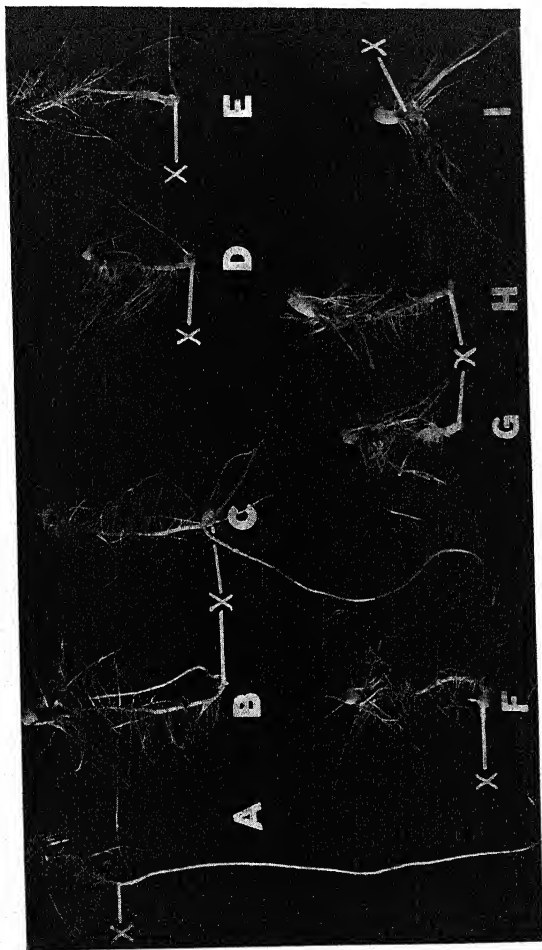


FIG. 5.—Primary roots, paste applied at *x*. *A*, *B*, *C*, *E*, *F*, *H*, *I*, 7 cm. long; *D*, *G*, 3 cm. long when treated. *I*, decapitated at *x*. *A*, water paste; *B*–*H*, heteroauxin paste; *B*, 5:10<sup>4</sup>; *C*, 2:10<sup>4</sup>; *D*, *E*, 1:2:10<sup>4</sup>; *F*, 2.5:10<sup>4</sup>; *G*, *H*, 3% paste. Note coil and swelling in *F*, *G*, *H*; swelling hyperhydric tissue breaking through epidermis; abnormally abundant laterals in all modified roots; resumption of growth, excepting at *G* and *H*.

laid on wet sand in flats, covered and kept in a dark room at 70°–80° F. and approximately 70 per cent humidity. Data were recorded after 24 and 72 hours. Photographs were made after five days (fig. 5). Sixteen plants were used for each dilution and for the controls. One set of roots (3 cm. long) was decapitated 4 mm. behind the tip.

The decapitated roots stopped elongation and promptly developed lateral roots in abundance (fig. 5I). The untreated and water paste roots grew vigorously (fig. 5A), the bend in the root being due to its contact with the wall of the flat. The controls had lengthened 30 mm. in 24 hours and 120 mm. in 72 hours (table 1, I).

The first macroscopically detectable effect was produced by concentration  $5:10^7$ . It produced positive right angle bending but no retardation (fig. 5B) was detectable by the technique used. Concentrations down to  $2:10^6$  produced positive bending and retarded elongation for several days (fig. 5B–H). Later, growth was resumed at a normal rate. Concentrations below  $4:10^6$  produced such sharp and repeated positive bending that in many cases coils resulted (fig. 5C–F) resembling snail shells. Even the coils resumed growth after several days. Concentrations 3 per cent,  $1:10^3$ , and  $1:10^4$  stopped elongation completely in some plants and induced local swelling at the tip (fig. 5G, H). After a few days these semi-spindles developed great masses of glistening white hyperhydric tissues which welled out from below the epidermis. All roots showing a right angle bend or more serious disturbances prematurely developed lateral roots up to the zone of injury (fig. 5B–H).

These results, especially the local tumors and the coiling, suggested legume nodules and the search for heteroauxin or similar substances in cultures of *Rhizobium phaseoli* and of mycorrhizal fungi.

**HYPOCOTYL.**—All dilutions were applied to the intact hypocotyl. In dosages of 0.01 and 0.0005 cc. of 3 per cent heteroauxin paste, response was equal the first few hours, proportional to the dose thereafter. Approximately 0.01 cc. 3 per cent heteroauxin paste applied unilaterally to hypocotyls before straightening of the arch produced negative curvature within a few hours; at times curvatures as great as 60° were noted (fig. 6B). Responses were most vigorous during the night. The next gross response was temporary retarda-

TABLE 1  
CHANGE IN ELONGATION (IN MILLIMETERS) DUE TO HETEROAUXIN PASTE APPLIED

CHANGE IN ELONGATION (IN MILLIMETERS) DUE TO HORMONE																		
ORGAN	CON- TROL*	WATER† PASTE	3%	1:10 <sup>4</sup>	5:10 <sup>4</sup>	2.5:10 <sup>4</sup>	1.2:10 <sup>4</sup>	6:10 <sup>5</sup>	3:10 <sup>5</sup>	1.5:10 <sup>5</sup>	7:10 <sup>6</sup>	4:10 <sup>6</sup>	2:10 <sup>6</sup>	1:10 <sup>6</sup>	5:10 <sup>7</sup>	2.5:10 <sup>7</sup>	1:10 <sup>7</sup>	AVER- AGE‡
I. Unilaterally to primary root tip																		
Root after 24 hrs.	21	28	0	0	0	0	0	0	0	0	0	22	20	25	26	28	29	25
Root after 48 hrs.	56	63	27	32	50	28	44	44	52	58	59	64	68	60	60	69	61	51
II. Unilaterally to hypocotyl																		
Hypocotyl.....	19	37	32	36	22	22	34	22	25	29	25	21	24	21	20	26	22	26
1st internode.....	54	45	48	47	56	53	36	36	50	51	45	57	61	54	64	82	58	53
III. By banding hypocotyl a. Green in light																		
Hypocotyl.....	15	36	25	26	28	38	35	35	35	30	31	22	8	.....	.....	.....	.....	30
1st internode.....	45	42	38	33	34	55	40	31	39	39	47	36	.....	.....	.....	.....	.....	39

\* Control means plants not altered experimentally. Values based on averages of at least 24 plants.

† Plants treated with water paste (1 part lanolin plus 1 part water) applied as indicated in the titles. Values based on only four plants for each experiment in V, VI, XI-XVI.

‡ Average means average change in elongation of organs in heteroauxin paste treated plants. If computed only on basis of usually effective concentrations (3% to 4:10<sup>6</sup>), the values for differences between water paste and heteroauxin paste treated plants would be more striking. Since only four plants were used for each concentration in experiments V, VI, XI-XVI, the numerical values for particularly significant concentrations cannot be given much quantitative weight. The organs of the bean being highly variable in length, repetition of the experiments using statistically significant numbers is desirable. While readings and measurements were made and recorded daily for a week, only the differences between initial and final measurements are given here. In many instances second and third day measurements are the most striking. The experiments extended from October to May, hence variations in lengths of organs in different experiments.

§ Concentration not tested.



TABLE 1—Continued

ORGAN	CON- TROL*	WATER† PASTE	3 %	1:10 <sup>3</sup>	5:10 <sup>4</sup>	2.5:10 <sup>4</sup>	1.2:10 <sup>4</sup>	6:10 <sup>5</sup>	3:10 <sup>5</sup>	1.5:10 <sup>6</sup>	7:10 <sup>6</sup>	4:10 <sup>6</sup>	2:10 <sup>6</sup>	1:10 <sup>6</sup>	5:10 <sup>7</sup>	2.5:10 <sup>7</sup>	1:10 <sup>7</sup>	AVER- AGE‡
III. By banding hypocotyl—Continued b. Etiolated in dark																		
Hypocotyl.....	160	80																
IV. To decapitated hypocotyl a. Green in light 1st experiment																		
Hypocotyl.....	4.8	7	4	6	5	2	4	9	2	4	Lost	4	4	4	3	4	4	4.4
2nd experiment																		
Hypocotyl.....	8.3	12	1	13	12	11	13	11	12	7	17							10
b. Etiolated in dark																		
Hypocotyl.....	5	9																
V. To wounds of decotyledonized plants																		
Hypocotyl.....	10	14	22	20	26	15	21	16	19	9	23	14	10	7	17	13	16	16
1st internode....	33	33	17	12	19	23	15	30	32	34	26	34	32	45	31	28	33	27
2nd internode...	5	4	0	2	3	6	1	2	3	4	3	5	5	6	4	4	5	3

TABLE 1—Continued

ORGAN	CON- TROL*	WATER† PASTE	3%	1:10 <sup>3</sup>	5:10 <sup>4</sup>	2.5:10 <sup>4</sup>	1:2:10 <sup>4</sup>	6:10 <sup>3</sup>	3:10 <sup>3</sup>	1.5:10 <sup>3</sup>	7:10 <sup>3</sup>	4:10 <sup>3</sup>	2:10 <sup>3</sup>	1:10 <sup>3</sup>	5:10 <sup>2</sup>	2.5:10 <sup>2</sup>	1:10 <sup>2</sup>	AVER- AGE‡
VI. To cotyledons on plants																		
Hypocotyl.....	31	17	25	29	34	27	26	27	21	24	20	20	15	20	25	28	29	25
1st internode....	66	61	33	52	37	58	50	63	77	63	59	56	62	58	56	57	60	62
2nd internode....	20	17	13	16	23	21	27	30	32	25	14	21	19	27	19	18	16	21
VII. To decapitated epicotyl 1st experiment																		
Hypocotyl.....	9	.....	.....	18	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
1st internode....	25	.....	.....	5	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
2nd experiment																		
Hypocotyl.....	32	32	32	18	17	22	19	24	20	20	30	26	30	26	27	23	32	24
1st internode....	8	8	8	9	8	13	12	17	8	8	9	8	11	10	7	10	10	10
VIII. By banding first internode																		
Hypocotyl.....	.....	6	10	13	15	4	13	9	4	5	9	13	18	9	7	3	.....	8
1st internode....	.....	36	30	23	30	22	32	39	30	35	36	31	49	36	35	34	.....	32.4
IX. Unilaterally to first internode																		
Hypocotyl.....	.....	5	8	15	17	8	12	4	11	15	2	9	9	10	Lost	3	Lost	9.4
1st internode....	.....	40	35	39	38	34	32	36	37	33	29	34	40	28	Lost	31	Lost	35

TABLE 1—Continued

ORGAN	CON- TROL*	WATER† PASTE	3%	1:10 <sup>3</sup>	5:10 <sup>4</sup>	2.5:10 <sup>4</sup>	1:2:10 <sup>4</sup>	6:10 <sup>5</sup>	3:10 <sup>5</sup>	1:5:10 <sup>5</sup>	7:10 <sup>6</sup>	4:10 <sup>6</sup>	2:10 <sup>6</sup>	1:10 <sup>6</sup>	5:10 <sup>7</sup>	2.5:10 <sup>7</sup>	1:10 <sup>7</sup>	AVER- AGE†
X. To decapitated epicotyl of decotyledonized plant																		
Hypocotyl.....	—	3	—	2	5	9	2	—	.5	—	2	—	1	0	—	2	7	1
1st internode.....	.....	6	5	6	6	5	10	7	5	7	5	5	5	6	11	6	6	7
XI. To decapitated petioles of second node																		
Hypocotyl.....	33	34	21	22	23	25	29	26	22	25	33	34	33	28	34	42	30	28
1st internode.....	32	28	35	40	38	30	45	42	34	37	31	35	25	28	31	28	27	34
2nd internode.....	13	14	16	28	12	10	21	18	18	14	11	15	23	8	12	13	13	15
Petioles.....	9	7	5	9	8	8	7	6	6	6	7	7	7	6	8	7	5	7
XII. To upper side of one petiole of second node																		
Hypocotyl.....	24	.....	27	20	29	27	22	16	25	17	19	18	19	.....	.....	.....	.....	22
1st internode.....	69	.....	43	62	53	58	69	55	65	67	65	66	36	.....	.....	.....	.....	58
2nd internode.....	11	.....	20	15	32	16	13	13	12	15	16	16	10	.....	.....	.....	.....	16
Untreated peti- ole.....	43	.....	51	49	49	46	53	49	50	49	45	43	38	.....	.....	.....	.....	47
Treated petiole.....	43	.....	28	45	40	40	47	39	50	50	45	44	45	.....	.....	.....	.....	43
XIII. To lower side of one petiole of second node																		
Hypocotyl.....	24	.....	32	20	17	22	32	24	25	9	30	42	23	.....	.....	.....	.....	34
1st internode.....	69	.....	37	47	57	61	59	61	55	43	46	60	61	.....	.....	.....	.....	57
2nd internode.....	11	.....	8	13	23	16	11	12	11	25	18	20	26	.....	.....	.....	.....	16
Untreated peti- ole.....	43	.....	37	43	57	53	54	47	55	47	45	38	43	.....	.....	.....	.....	46
Treated petiole.....	43	.....	17	31	47	35	43	43	50	43	38	41	45	.....	.....	.....	.....	50

TABLE 1—Continued

ORGAN	CON- TROL*	WATER† PASTE	3%	1:10 <sup>3</sup>	5:10 <sup>4</sup>	2.5:10 <sup>4</sup>	1.2:10 <sup>4</sup>	6:10 <sup>5</sup>	3:10 <sup>5</sup>	1.5:10 <sup>5</sup>	7:10 <sup>6</sup>	4:10 <sup>6</sup>	2:10 <sup>6</sup>	1:10 <sup>6</sup>	5:10 <sup>7</sup>	2.5:10 <sup>7</sup>	1:10 <sup>7</sup>	AVER- AGE‡
XIV. By banding one petiole of second node																		
Hypocotyl.....	24	.....	38	22	17	17	19	17	27	33	21	20	15	.....	.....	.....	.....	23
1st internode....	69	.....	41	33	62	45	48	47	55	70	53	59	50	.....	.....	.....	.....	52
2nd internode....	11	.....	7	11	12	38	16	20	20	15	22	22	22	.....	.....	.....	.....	16
Untreated peti- ole.....	43	.....	41	45	52	51	45	57	50	48	43	43	47	.....	.....	.....	.....	47
Treated petiole.	43	.....	22	17	30	41	38	43	44	50	48	47	53	.....	.....	.....	.....	39
XV. To upper side of one leaf of second node																		
Hypocotyl.....	24	.....	7	37	19	19	18	15	17	21	21	24	17	.....	.....	.....	.....	20
1st internode....	69	.....	35	58	72	63	63	49	69	60	59	49	53	.....	.....	.....	.....	57
2nd internode....	11	.....	6	15	22	22	11	26	26	16	18	14	16	.....	.....	.....	.....	17
Petiole untreated leaf.....	43	.....	48	45	60	50	52	.....	57	.....	.....	.....	.....	.....	.....	.....	.....	52
Petiole treated leaf.....	43	.....	32	45	57	50	50	56	47	50	50	43	42	.....	.....	.....	.....	47
XVI. To lower side of one leaf of second node																		
Hypocotyl.....	24	.....	25	20	29	17	39	15	17	22	20	15	17	.....	.....	.....	.....	22
1st internode....	69	.....	25	45	53	45	48	49	49	60	47	45	53	.....	.....	.....	.....	47
2nd internode....	11	.....	3	15	32	18	16	26	9	16	3	20	16	.....	.....	.....	.....	15
Petiole untreated leaf.....	43	.....	35	51	.....	.....	.....	.....	50	.....	33	.....	.....	.....	.....	.....	.....	42
Petiole treated leaf.....	43	.....	16	40	49	51	45	50	47	52	33	53	49	.....	.....	.....	.....	44

tion of hypocotyl elongation and of epicotyl unfolding (fig. 6*B*), although in time the treated plants attained hypocotyl length greater than normal. Whitening at the site of application appeared in one to two hours, depending upon the concentration and amount of paste used. After several hours definite swelling developed at and below the site of application (fig. 6*B*), extending downward below the soil line. (When after the application of 3 per cent heteroauxin paste to the hypocotyl, incisions were made and glass plates in-

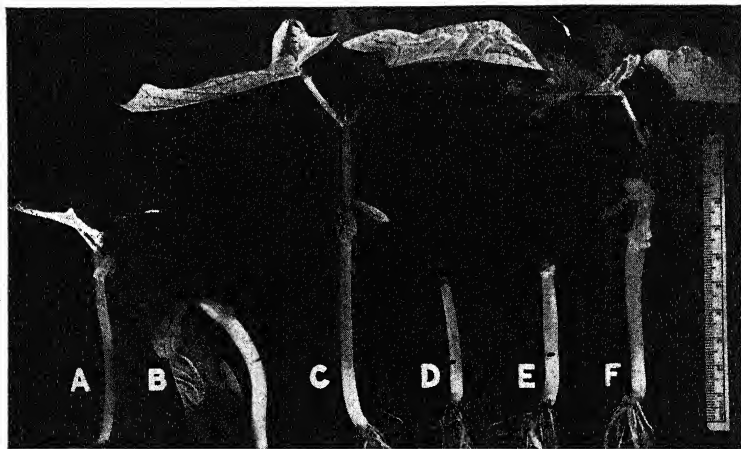


FIG. 6.—*A*, control; *B*, seedling treated unilaterally with  $1:10^3$  heteroauxin paste; photographed after 6 hours. Note negative bending, retardation of elongation and of unfolding of epicotyl, swelling of hypocotyl greatest at and below site of application. Black line in each specimen indicates soil line. *C*, control; *D*, decapitated hypocotyl with water paste; *E*, decapitated hypocotyl with  $1:10^3$  heteroauxin paste. Note local swelling, whitening, and general diameter increase. *F*, hypocotyl banding with  $1:10^3$  paste. Note diameter increase of hypocotyl and first internode, lengthening of hypocotyl and shortening of first internode.

serted, directly below the application at hourly intervals effects were found to have passed below that point in two hours or less.) Later the hypocotyl also swelled along all radii, from a short distance above the site of application to the soil line. The average increase in diameter was 20 per cent. If application was made near the cotyledons, the first internode also swelled. Finally the site of application was marked by a colorless intumescence which projected above the level of general swelling (fig. 7*A*). The new tissues were

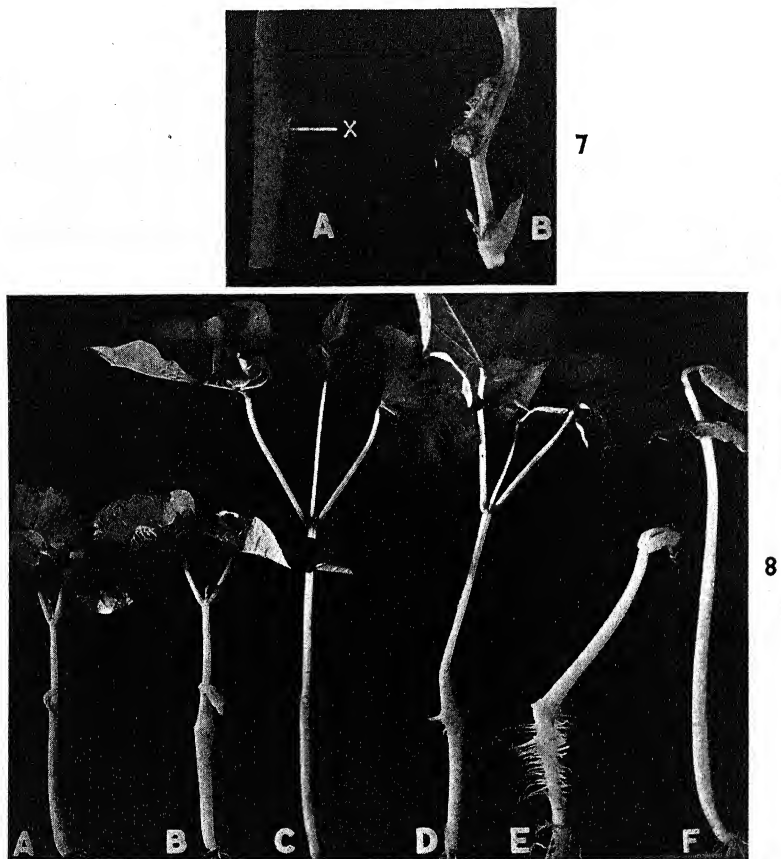
pathic. This was evident in the observation that during fumigation with hydrocyanic gas they were killed while the non-injured tissues were not affected. In moist air these tissues molded and in dry air they desiccated more rapidly than did the non-treated tissues.

The treated region developed abundant adventitious roots which did not emerge unless the air was quite moist (fig. 8C, D). No difference was found in the number of roots produced in reference to point of application on the hypocotyl circumference, whether on a line under or between the cotyledons.

Paste was also applied by banding the hypocotyl just below the arch (figs. 6F; 8B, E; 9A). Usually this did not produce bending, but was followed by more pronounced expression of the symptoms noted for unilateral applications. Generally banded hypocotyls attained greater length than controls (figs. 6C, F; 8A, B); at times the higher concentrations, especially 3 per cent, repressed elongation. Banding of the hypocotyl shortened the first internode (figs. 6C, F; 8A, B). The highest dilution effective for the hypocotyl was 4:10<sup>6</sup> irrespective of mode of application. While the treatment increased diameter as well as later elongation, the final length of the hypocotyl usually was about that of a normal plant (table 1, II, III).

Age of the area to which heteroauxin was applied was significant. When only 1 cm. long, or directly below the cotyledons while still arched, the hypocotyl was unresponsive. Hypocotyls ten days old were markedly less reactive than those eight days old. When 15 days old they gave no response unless wounded. That this is not due to loss of reactive capacity by the cells but presumably to increased impermeability of the aged epidermis was demonstrated by applications of 3 per cent paste to old hypocotyls or internodes incised longitudinally. In moderately dry air these developed massive calluses full of root primordia. Placed in moist air, enormous root development took place (fig. 9B). In a few plants secondary tumors as well as roots apparently developed distally to the site of application. Great care had been taken to prevent spread of the inoculum. Freehand sections revealed continuity between the main and the smaller swellings. Frequently roots arising near the site of injury elongate downward just below the epidermis, without emerging.

**DECAPITATED HYPOCOTYLS.**—Decapitated hypocotyls develop surface callus tardily. If still young at the time of transection they elongate for a few days and then contract. This shortening was



FIGS. 7, 8.—Fig. 7, *A*, hypocotyl 8 days after unilateral treatment with 1:10<sup>3</sup> paste. *x* is site of sections of figs. 21 and 22. Smaller amounts of paste produced smaller involved areas. Sections of figs. 21 and 22 prepared from hypocotyl treated with much smaller amounts. *B*, second internode treated with 1:10<sup>3</sup> paste, kept in moist air. Note roots. Fig. 8, *A*, control; *B*, hypocotyl banded with 1:10<sup>3</sup> paste; kept in moderately dry air. Note greater thickening and elongation. *C* and *D* kept in very moist air. *C*, control; *D*, hypocotyl treated unilaterally with 1:10<sup>3</sup> paste. Note roots at and below site of treatment. *E*, *F*, etiolated plants. *F*, control; *E*, hypocotyl banded with 1:10<sup>3</sup> paste. Note bending, swelling, decreased elongation, retardation of epicotyl development, abundant roots in four rows.

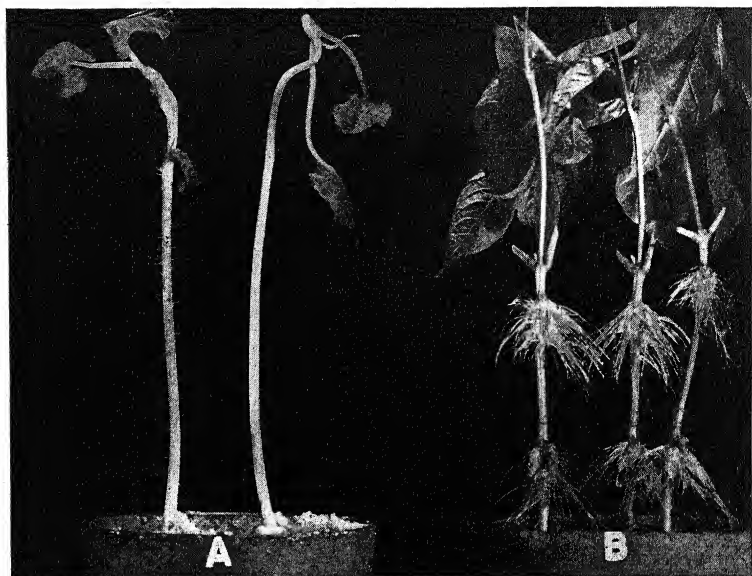
noted in all hypocotyls measured. Treatment of the surface with control paste did not affect the elongation but led to more callus development. Heteroauxin paste led to increased elongation, to diameter increase, to swelling at the tip, and to root formation (fig. 6*D*, *E*; table 1, IV). Concentrations down to  $7:10^6$  produced general diameter increase and local swelling. Concentrations below  $2.5:10^4$  produced whitening below the site of application as well as initial constriction immediately below the treated surface. Roots were produced by concentrations of  $7:10^6$  and greater. They were sparingly produced in dry but abundantly in moist air, developing in and below the tumors.

**ETIOLATED HYPOCOTYL.**—Unilateral and band applications to the etiolated seedlings in concentrations 3 per cent and  $1:10^3$  definitely retarded elongation (fig. 8*E*, *F*). No other dilutions were tried. Applications to the decapitated hypocotyl definitely increased elongation over that of the decapitated control, which elongated very slightly. Unilateral applications were followed by negative bending; banding applications by no bending. The tumors produced attained large size, 100 per cent increase over the normal hypocotyl diameter not being exceptional. Band applications at any level of the hypocotyl led to development of four rows of roots below the site of application down to the soil level (fig. 8*E*). If the first internode was banded, roots developed below the band on the first internode and on the hypocotyl (fig. 9*A*). Unilateral applications led to root development at the site of application and on the side immediately below (table 1, III*a*, IV*b*).

**SEGMENTS OF HYPOCOTYL.**—Treated segments of the hypocotyl showed whitening, swelling, root and callus production at the surfaces, and apical as well as basal roots. The only concentration used was 3 per cent.

**DECOTYLEDONIZED SEEDLING.**—Applications of paste to removed cotyledon sites in dilutions up to  $4:10^6$  led to diameter increase of the hypocotyl. Unfolding of the epicotyl was retarded by dilutions up to  $6:10^5$ . This treatment increased elongation relative to decotyledonized water paste plants (fig. 10*C*, *D*) but this increase did not equal that of the normal plant (fig. 10*A*, *B*, *E*; table 1, V).





9



1

FIGS. 9, 10.—Fig. 9, *A*, etiolated plants. To left, epicotyl banded with  $1:10^3$  paste. (This experiment first performed by Dr. JOHN W. MITCHELL of this department.) Note swelling and retarded elongation of first internode, roots at site of tumor and in four rows throughout entire length of hypocotyl. (Tops of plants bent by bell jars.) *B*, plants grown in moderately dry air; hypocotyl and first internodes ceased elongation. Upon unilateral application of 3% paste no gross responses occurred. Longitudinal incision of hypocotyl and first internode and application of 3% paste there resulted in whitening, in an enormous callus which lengthened and widened the slit, and in roots mostly in four rows (if plants were kept in moist air). Roots established themselves in soil and became functional. In drier air, roots did not emerge. Fig 10 *E*, control plant. *A*, *B*, decotyledonized plants; *C*, *D*, decotyledonized and wounds treated with  $5:10^4$  paste. Retarded development in *A* and *B* but increase in *C* and *D*; greater diameter increase in *C* and *D*. Cotyledons removed with razor blade. For paste treated cotyledons, see fig. 3*G*.

**SEVERED COTYLEDONS.**—Since cotyledons occasionally develop roots after removal from the plant, the cotyledons of the previous experiments were kept in pairs, one of each pair being treated with the paste. They were then placed on moist sand in the dark. Cotyledons pulled from the plants are more likely to develop callus and roots than those cut from the plant. The former procedure probably removes more of the active part of the cotyledon from the axis. Of 30 non-treated cotyledons, only two developed roots (fig. 3*F*) while 14 of 30 so treated developed roots (fig. 3*G*). Concentrations 3 per cent and  $1:10^3$  to  $1.5:10^5$  were effective. All roots were vigorous excepting those produced by concentration  $1.5:10^5$ . Many cotyledons did not develop roots until eight or nine days after removal and treatment.

**TREATED COTYLEDONS.**—Cotyledons on young plants were treated with pastes of all concentrations. On the average, treatment increased the length of both the hypocotyl and of the first internode and delayed abscission of cotyledons (fig. 11; table 1, VI).

**DECOTYLEDONIZED AND DECAPITATED FIRST INTERNODE.**—Treatment with pastes of wounds resulting from these operations led to general diameter increase (10 per cent) of the first internode and hypocotyl in dilutions up to  $4:10^6$ . In the stronger concentrations, local swelling at the cut (20–40 per cent) as well as massive surface callus developed. Whitening developed immediately below the site of application in the stronger concentrations (fig. 12*A–C*). On the average, treatment increased elongation of the hypocotyl (table 1, X).

**UNILATERAL APPLICATION TO, AND BANDING OF, FIRST, SECOND, AND THIRD INTERNODES.**—Unilateral application of the pastes to the internodes led to bending, retardation of elongation, and local swelling in dilutions up to  $4:10^6$  (figs. 13*A, D*; 14*D*). The hypocotyl was lengthened. Concentrations 3 per cent and  $1:10^3$  produced whitening (fig. 13*A, B*). Roots developed from the tumors (fig. 7*B*). Banding of first internode led to temporary retardation, then greater ultimate elongation of the hypocotyl and shortening of the first internode. The end point of concentrations was the same as in unilateral application. Other effects were identical except bending (table 1, VIII, IX).

DECAPITATED FIRST INTERNODE.—Treatment of decapitated first internodes decreased hypocotyl elongation but increased diameter increase (fig. 12*D*, *E*). The end point of reaction was dilution  $7:10^6$  (table 1, VII).

DECAPITATED SECOND AND THIRD INTERNODES.—Decapitation of these internodes was not followed by elongation. Under moist conditions a surface callus developed (fig. 13*H*). Under the usual condi-

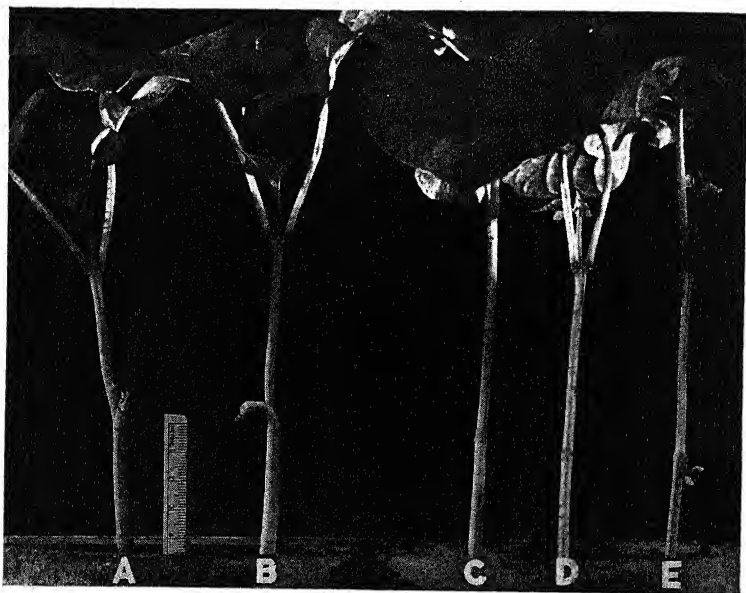


FIG. 11.—*A*, *B*, cotyledons treated with  $5:10^7$  paste; *C*, *D*, *E*, control plants. Note increased elongation of hypocotyl and retention of cotyledons in *A*, *B*. *A* and *B* show method of measurement: a gardener's label was placed on the pot and measurement of hypocotyl was made from top of label. Most controls used during May appeared like *C*, *D*, and *E*.

tions prevailing during the experiments no surface callus developed (fig. 13*I*). Application of pastes up to dilutions  $4:10^6$  produced local swelling and surface callus (fig. 13*E-G*). General diameter increase ranging from 10 to 20 per cent was produced by 3 per cent and  $1:10^3$  paste. Roots developed abundantly from the surface callus and the swelling (fig. 13*E*), especially in moist air.

SECOND NODE.—Young petioles severed immediately below the blade pulvinus were treated with the pastes (fig. 12*F-H*). In dilu-

tions up to  $4:10^6$  the petiole increased in diameter, swelled locally at the cut, and developed surface callus. Length of the hypocotyl was increased over that of water paste treated plants (fig. 12H)

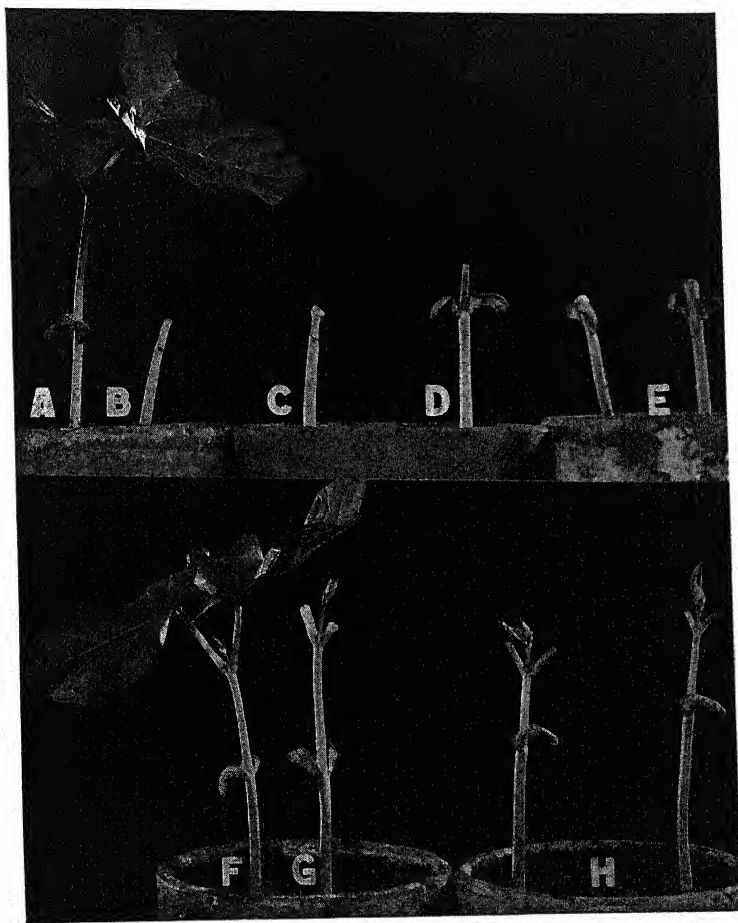
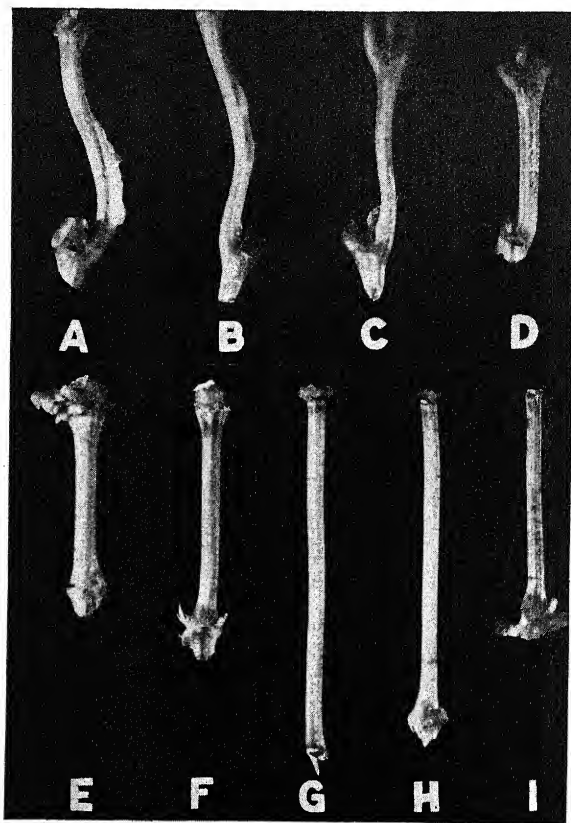
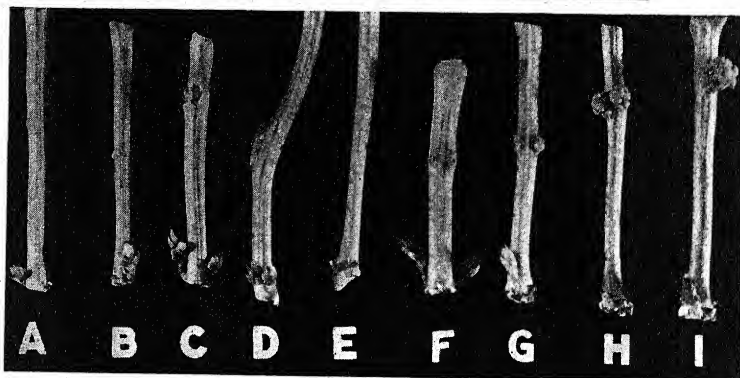


FIG. 12.—A, control; B, decotyledonized plant with decapitated first internode. C, same treated with  $3:10^5$  paste. Note local swelling of first internode and general thickening of hypocotyl. D, decapitated first internode. Note increase in length and of diameter of hypocotyl. See fig. 4D for nature's experiment. E, decapitated first internode treated with  $1:10^3$  paste. Note diminished elongation of hypocotyl, first internode, as well as general diameter increase and local swelling due to concentrated paste. F, control; H, decapitated petioles; G, same treated with  $1:10^3$  paste. Decapitation of petioles resulted in increased length of hypocotyl and of second internode. Application of paste shortened hypocotyl, lengthened first and second internodes.



13



14

FIGS. 13, 14.—Fig. 13, A–C, second internodes treated with paste. A, 3%; B, 1:10<sup>3</sup>; C, 4:10<sup>6</sup>; D, water paste; E–I, decapitated second internodes. E, treated with 3% paste; F, with 1:10<sup>3</sup>; G, with 7:10<sup>6</sup>; H, I, with water paste. Note surface callus in E–I, local swelling, general diameter increase and roots in E and F. Fig. 14, treated second internodes. A, water paste; B, ether extract of non-inoculated dextrose-tryptophane medium; C, ether extract of dextrose-tryptophane culture of *P. tumefaciens* (taken after internode had straightened after initial bending). Note central sunken area due not to killing but to locally inhibited growth. D, 1:10<sup>3</sup> paste; E, *P. tumefaciens* applied without puncture; F, needle puncture; G, 2 weeks old gall following inoculation of *P. tumefaciens* by puncture; H, 8 weeks old callus at puncture site; I, 8 weeks old gall at puncture site.

whose hypocotyl was longer than that of controls (fig. 12*F*). Length of the first and second internodes was increased by petiole decapitation (fig. 12*H*). These results indicate inhibiting effects of leaves on elongation of the axis below, and of the growing region above them. Treatment with paste further increased differences in lengths of hypocotyls and second internodes (table 1, XI).

APPLICATION TO UPPER AND LOWER SIDES AND BANDING OF PETIOLES OF SECOND AND OTHER NODES.—Dilutions up to  $7:10^6$  produced negative bending as well as local swelling. Higher concentrations also produced whitening (fig. 15*A*, *C*). Application of paste to the upper and lower sides of petioles led to slight lengthening of the hypocotyl, first and second internodes. Treatment of upper side led to shortening, while treatment of lower side led to lengthening of the petiole.

Banding of the petiole led to local swelling, to assumption of the vertical position by the petiole, and to decreased elongation. Concentration  $4:10^6$  was the end point. The higher concentrations produced whitening (fig. 15*B*; table 1, XII, XIII, XIV).

PULVINI AT BASE OF LEAF BLADE.—Treatment of either the upper or lower side of the pulvini of leaves of the second node resulted in negative bending (fig. 16). Concentrations  $7:10^6$  and greater were effective. In treatment of the lower side of the pulvinus higher concentrations turned the leaves completely back so that the lower surface became the upper (fig. 16*A*, *C*, *D* righthand). Treatment of the upper side with high concentrations turned the leaves downward past the vertical position (fig. 16*D*, lefthand).

LEAF BLADES.—Leaves of the second node were treated by application of strips of paste across the middle of the leaves from margin to midrib or across the entire width on the lower or upper side. In young leaves dilutions up to  $7:10^6$  and in older leaves up to  $1.5:10^5$  diminished elongation of the petiole (fig. 15*F*); treatment of the lower side increased elongation (fig. 15*E*; table 1, XV, XVI). In all cases treatment caused upward folding along the midrib (fig. 17*A*, *B*). In concentrations above  $6:10^5$  torsions of the petioles developed in leaves treated only on one-half the blade. The treated half of the leaf was turned up vertically or even to a new horizontal position with the lower side uppermost.



In younger leaves, that is, leaflets of the third node (fig. 17C-E), the effects were essentially the same with additional symptoms.

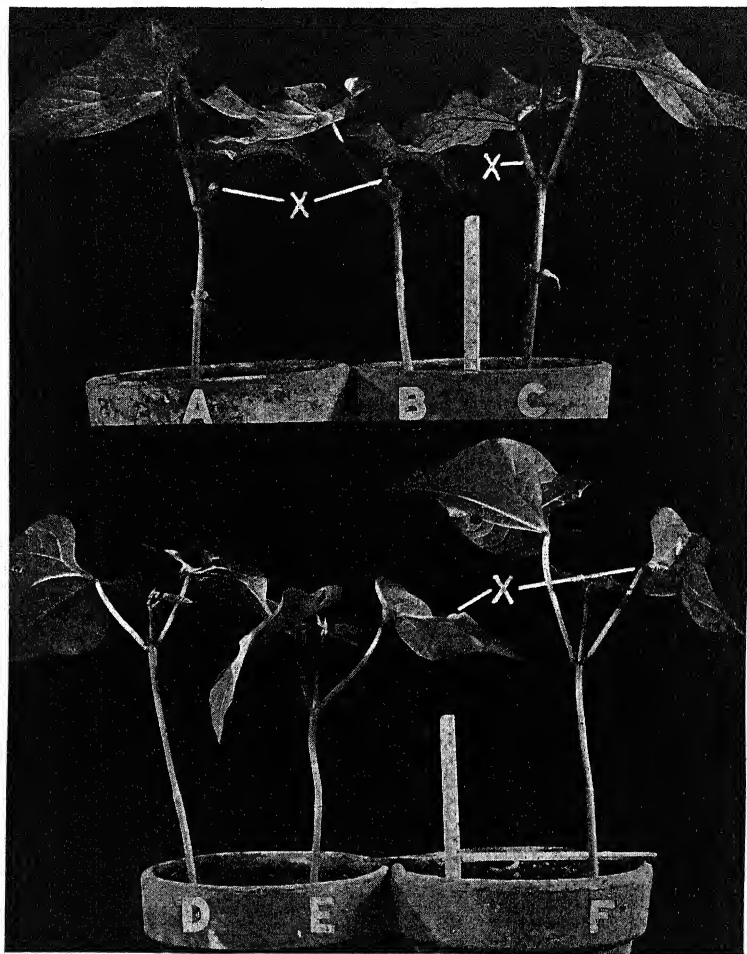


FIG. 15.—A-C, petioles treated with paste. A, petiole treated with 2.5:10<sup>4</sup> paste on upper side at *x*; B, treated at lower side at *x*; C, banded at *x*; D, control; E-F, leaves treated; E, leaf on right hand treated on lower side. Note elongated petiole, *x*. F, leaf on right hand treated on upper side, petiole shortened, *x*.

The form of such leaves was affected. At and beyond the site of application the leaf was less expanded than normally or there was

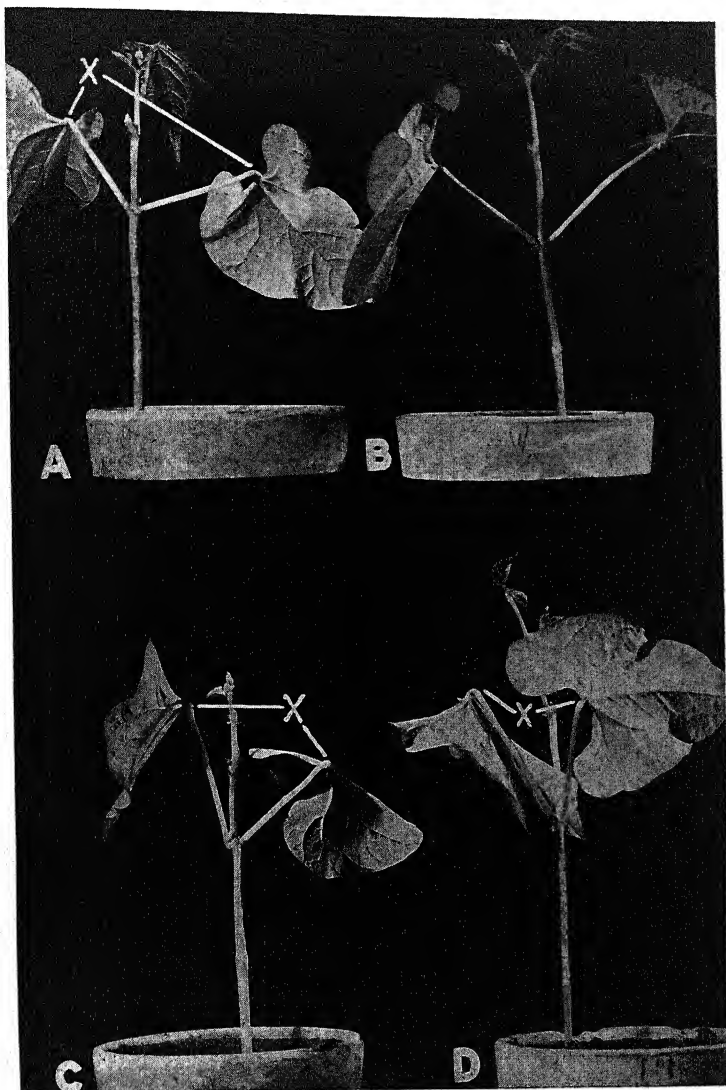


FIG. 16.—A, C, D, pulvini of lefthand leaves treated on upper side at  $x$  with heteroauxin paste; pulvini of righthand leaves treated on lower side at  $x$ . A, 1.2: 10<sup>4</sup>; C, 1: 10<sup>3</sup>; D, 3% paste. B, pulvini treated with water paste. Note that lower sides of leaves in A, C, D, right, have become the upper sides due to torsions in petioles.



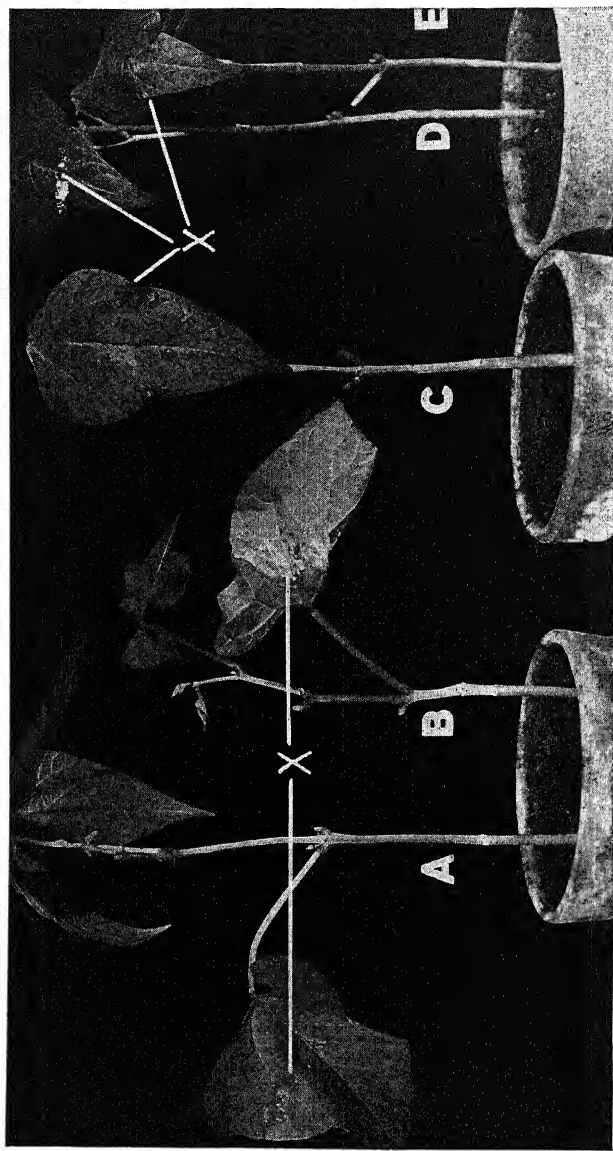


FIG. 17.—Leaves banded with paste on one side at  $\times$ . A, 3% paste upperside. Note upward folding. B, 1:10<sup>3</sup> paste on lower side. C, third node leaflet, 2.5:10<sup>4</sup> paste applied to lower side. Note constriction at site of treatment, puckering, sawing, mottling, and downward bending at site of treatment. D, E, 1.2:10<sup>4</sup> paste applied to lower and upper sides respectively. Effects as in C.

merely a constriction at the site of treatment. This occurred in concentrations  $6:10^5$  and greater. The surfaces of such leaves were savoyed and puckered, and all imaginable types of rolling and curling, as well as under-development, were noted comparable to those characteristic of the pseudogalls incited by leaf insects. The color below the strips changed, ranging from a mosaic of different shades of green to mosaics of green and yellow (fig. 17C-E). The same results were obtained on young leaves of the second node. In many young leaves the treatment caused negative bending of the midrib resulting in upward or downward flection (fig. 17C-E) of the distal half of the leaf. The affected part of the midrib developed tumors with roots, especially at concentration  $1.2:10^4$ .

Young and aging leaves treated with 3 per cent paste yellowed and dried prematurely. This and premature death of decapitated and treated hypocotyls are the only instances of gross lethal effects from treatment with chemically pure heteroauxin.

Studies of the effects of higher concentrations on various parts of the bean and of various concentrations on the germinating seed are under way.

#### TOMATO

In preliminary experiments with 2 and 3 per cent paste, young tomato plants showed whitening, diameter increase, and prompt adventitious root development proportional to the concentration. Decapitated top internodes showed similar responses. Further experiments were made on tomato seedlings and older plants. The tomato is not so responsive either in rate or amount to heteroauxin as is the bean. Dilutions above  $1.5:10^5$  produced no gross responses in aerial parts, whereas the bean reacted to dilutions to  $4:10^6$ . No experiments were made on tomato roots.

Hypocotyls treated with pastes applied unilaterally in dilutions up to  $1.5:10^5$  bent negatively, swelled locally, but gave little diameter increase. Whitening was less marked than in the bean. Root development, however, was more active. Decapitated hypocotyls swelled locally, increased in diameter, and developed surface callus.

Epicotyls treated in the first, second, third, and fourth internodes, as well as petioles, reacted as did the hypocotyl to unilateral ap-

plication or following decapitation. Band applications produced the same effects but no bending.

Application of pastes to the stem produced not only tumors and roots, but epinastic growth of the leaves as well (fig. 18B). In the latter respect the tomato is more reactive than the bean.

Tomato seedlings grown in sand on a nitrogen-free nutrient solution had ceased growth at time of application with 3 per cent paste. Whitening and later local swelling set in, and growth continued for a considerable period, the tumor acting as a new growth region.

#### TREATMENT WITH AGAR CULTURE BLOCKS

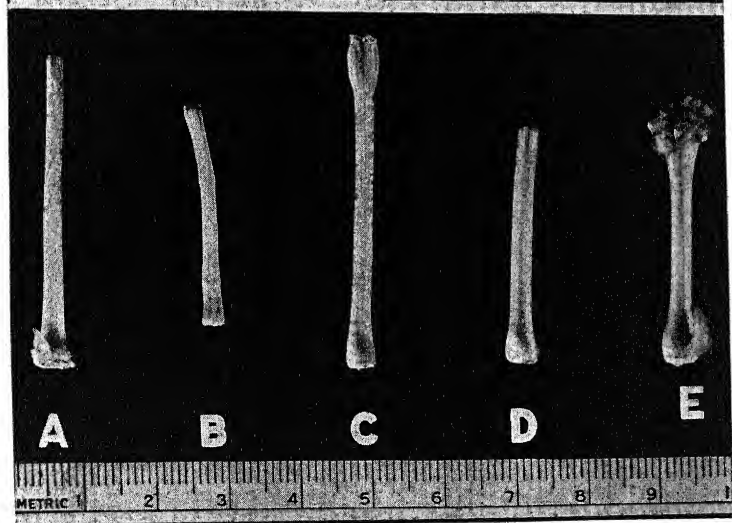
Blocks of three months old dextrose-tryptophane and dextrose-tryptophane and peptone and two days old dextrose potato agars on which *P. tumefaciens* had been cultured were used in these experiments. Although thoroughly rinsed, no attempt was made at sterilization. Each block was fastened to the surface of decapitated bean hypocotyls by means of fine glass capillaries run through the agar into the hypocotyl. All agars on which *P. tumefaciens* had grown produced swelling at the tip of the hypocotyl. In some instances the first effect was constriction at the very top. The most pronounced growth effects were produced by the dextrose tryptophane agar and the least by the potato dextrose agar. (Possibly this was due to the youth of the culture.) Uninoculated control agar blocks were negative. Cultures of *P. tumefaciens* applied to the surfaces of decapitated hypocotyls caused similar results but less rapidly. Obviously *P. tumefaciens* either liberates a growth substance into its culture medium, or by liberating enzymes changes the ingredients of the medium into growth substances.

#### EXTRACTS OF *P. TUMEFACIENS*

Aged cultures of *P. tumefaciens* grown on potato dextrose agar in Kolle flasks, then treated with merthiolate or ether extracted, gave faint if any reaction when applied to young tomatoes. All extracts from broth culture were active but varied greatly in strength, as would be expected in such crude preparations. Three weeks old cultures were less potent than those three months old. This activity has been retained during the six months period since their prepara-



18



19

FIGS. 18, 19.—Fig. 18, *A*, control plant grown in dry air and then placed in Wardian case with high humidity; *B*, plant treated identically but inoculated at  $\alpha$  with 1:10<sup>3</sup> heteroauxin paste. Note tumor, epinasty, roots, retention of leaves. Identical effects obtained with young tomato plants treated with extract of *P. tumefaciens*. Control extract produced no effects. Fig. 19, decapitated second internodes of bean. *A*, control; *B*, treated with extract of non-inoculated dextrose-tryptophane medium; *C*, treated with extract of *P. tumefaciens* grown in dextrose-tryptophane medium. Note swelling, diminished near surface. *D*, treated with water paste; *E*, treated with 1:10<sup>3</sup> heteroauxin paste. All kept in moderately moist air for 3 weeks.

tion. The most active, and consequently used for most experiments, was the extract of the three months old culture in the medium without peptone. It caused pronounced curvature of *Avena* coleoptiles and gave a marked pink in the specific test for heteroauxin. On tomato seedlings it caused local killing, extensive diameter increase, local whitening, and positive bending when applied to the first internode. Roots appeared here as swellings below the surface and at midribs and lateral veins of inoculated leaves. Stem applications caused similar responses in larger tomatoes. When applied to decapitated apical internodes, epinastic bending of leaves ensued.

Application to the second internode of the bean resulted in whitening, diameter increase, root production, and usually positive bending (fig. 14C, taken after internode had straightened). Negative bending occurred only in two cases. In the young hypocotyl similar effects ensued, although the bending was always negative (fig. 20C). Elongation was much retarded as in the application of 3 per cent heteroauxin paste. Treatment of decapitated hypocotyls and internodes led to responses not noted after application of heteroauxin—a real or apparent constriction at the surface of the stump followed by swelling below and later by less rapid and extensive diameter increase in the constricted zone (fig. 19C). The end results resembled those obtained by CZAJA (7) in decapitated *Helianthus* hypocotyls treated with heteroauxin and water paste in different concentrations and combinations. The ether extract of the non-inoculated medium produced no effects when applied to plants (figs. 14B, 19B, 20B).

These experiments are less comprehensive than those with heteroauxin. The results are not directly transferable because the extracts may contain active substances other than heteroauxin. Application of some of the extracts to apical tissues of the tomato resulted in killing, and on young internodes of the bean in temporary positive bending, indicating either that the concentration of the heteroauxin is greater than 3 per cent or that other more effective substances are present. Sections of internodes with positive bending reveal no killing of tissues, indicating that the young bean internodes are less sensitive than those of the tomato to killing by some ingredient of the extracts. The wounding experiments with acetic acid indicate



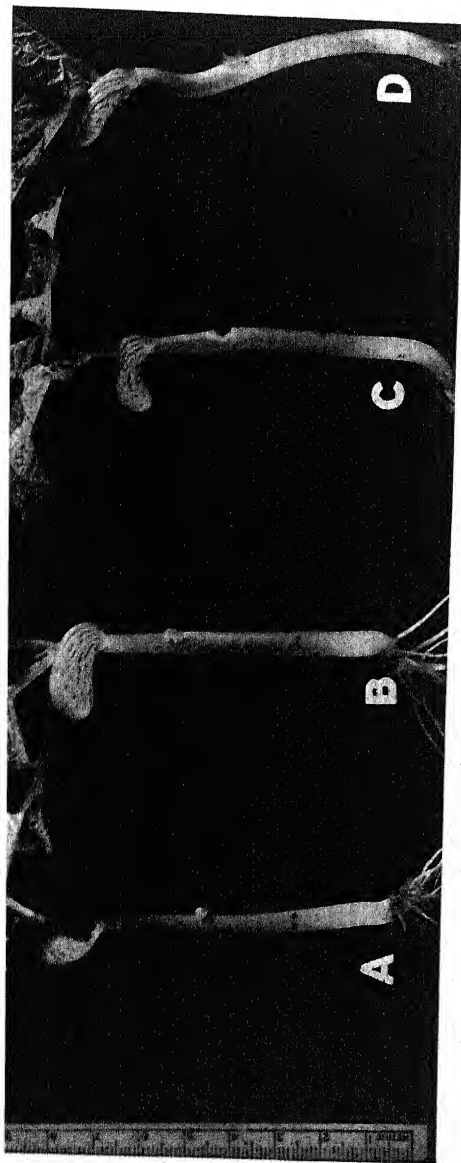


FIG. 20.—A, control hypocotyl treated unilaterally with water paste. B, control treated unilaterally with extract of non-inoculated dextrose-tryptophane medium. C, treated with extract of dextrose-tryptophane culture of *P. tumefaciens*. Black material is the extract. Note local depression due not to killing but to locally lessened swelling, peripheral swelling and whitening, general diameter increase, retarded elongation of hypocotyl, and retarded expansion of epicotyl. Photographed after 12 hours when initial bending had been almost rectified. Sections of figs. 21 and 22 cut from such a site after 8 days. D, unilateral application of 3% paste after 10 hours. Note bending being rectified, diameter increase, local swelling, whitening, retardation of epicotyl development. Black line indicates soil line.

that the primary growth responses incited by the extracts are initial growth responses to these substances and not secondary growth responses incited by local necrotization.

In initial experiments the extracts were over-diluted in lanolin, as indicated by feeble responses of the plants. From later experiments and written information from Dr. F. E. GARDNER, it is further concluded that these poor results were also due to age of test plants and unfavorable growing conditions. In later experiments mixture of the extracts with lanolin before application might have extended their period of activity.

#### TREATMENT WITH GALL EXTRACTS

Chloroform extracts of *P. tumefaciens* incited tomato galls, and control extracts of the stem and leaves when applied to the bean caused local killing. The gall extract when applied with puncture caused slight local swelling and whitening on the hypocotyl. On the second internode it produced slight swelling and slight positive bending; stem and leaf extracts were negative. On tomato seedlings killing and doubtful negative bending occurred. On older tomatoes killing was the only noted response. These tests were all essentially negative. They are being repeated.

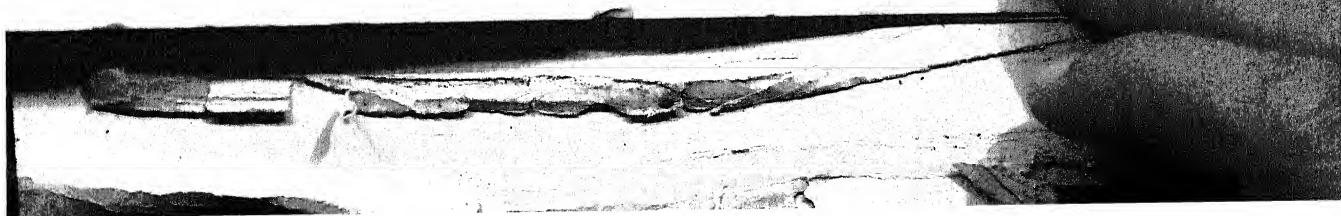
#### HISTIC STUDIES

Samples of tumors induced by bacterial extracts and by 3 per cent heteroauxin paste were collected at frequent intervals and placed in Navashin's solution preliminary to histic studies. This paper will deal only with eight days old material produced by treatment with the extract. Treatment with 3 per cent heteroauxin paste when applied in dosage of 0.005 cc. produces essentially the same effect. The sections reproduced in figures 21C and 22B, D are from tumors such as shown in figures 7A and 20C. The hypocotyl was inoculated just below the arch before straightening. In the mature hypocotyl the lesion finally was 2-3 cm. above the soil level. The gross symptoms are those discussed in the section on treatment of the hypocotyl.

Initial bending of the hypocotyl is due to cell enlargement, most pronounced on the treated side and along the radial and tangential axes of the cells; cortex, including the endodermis, and the rays and

pith are most affected. While the effect was most marked in the cortex, under and adjoining the site of application, the entire hypocotyl was affected. This was evident in change opposite the site of application (fig. 22A, B). Clearing is due to disappearance of chlorophyll, but even more to increase in volume of the intercellular spaces of the cortex (figs. 21C; 22B, D). This increase was brought about by such marked enlargement of cells of the cortical parenchyma that some adjoining cells began to pull apart at their middle lamellae (fig. 21C). The resultant tissues are identical with the chemically induced pathic tissues which KÜSTER (17) classified as hyperhydric. In the latter stages of intumescence complete loss of continuity between cells of the cortical parenchyma ensued. Meanwhile cortical cells adjoining the endodermis and the endodermal cells enlarged greatly radially and less tangentially, and began to divide. Division was mostly tangential, but also radial and less frequently oblique, the new walls being laid down within the greatly stretched old walls (fig. 22A-D). Other cortical cells divided and the resultant tissue resembled an internal callus. The nuclei of the affected cells were greatly enlarged.

The cells of the cambium, primary phloem, and rays are likewise characterized first by dimensional, later by numerical increase. Eventually the phloem gave rise to new meristems and bundles as described for decapitated and heteroauxin treated second internodes of *Phaseolus vulgaris* (16). The pericycle appeared relatively inactive. Cells bordering the cavity, including the endodermal cells or their tabular derivatives, at times developed as long, hairlike cells into the cavity (fig. 21C). These cells simulated the much elongated cells characteristic of many foliar intumescences (17) and of surface callus. Other cells bordering or distant from the cavity at times divided in various planes. Continuity of the epidermis was rarely broken, apparently because of continued radial divisions (fig. 22B, D). Cortical cell tissue, including the endodermis, on occasion produced the isolated or grouped, short, thick walled elements which KÜSTER (17) designated wound tracheids. The endodermis and adjoining cortical cells at times gave rise to islands of meristems with resultant extrafascicular vascular bundles as described for incised internodes of *P. multiflorus* by SCHILBERSKY (29) and for de-





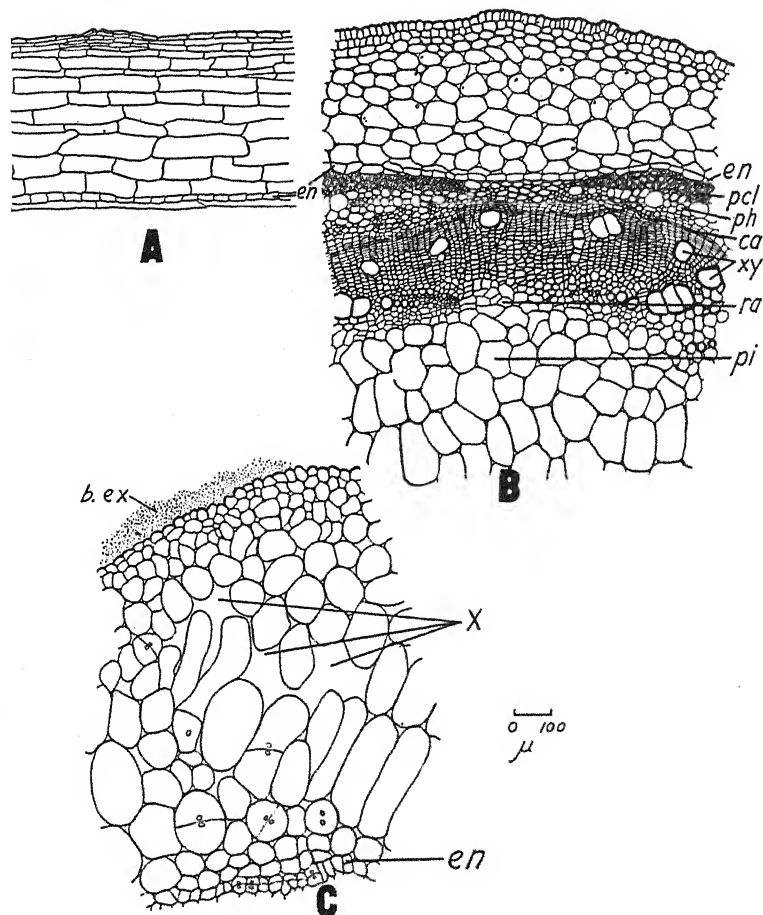


FIG. 21.—*A*, longitudinal section of cortex of normal hypocotyl of bean; *B*, transverse section at same level as figs. 21*C* and 22*B*, *D*. All same age. Secondary thickening and development of thick walled elements in *B*. *C*, transverse section through region of hypocotyl treated with bacterial extract (*b.ex*) fixed 8 days after treatment. Cell division in epidermis and subepidermal layers, more pronounced under paste; less thickening of axis immediately under paste; enlargement of most cortical cells, division in endodermis (*en*) and cortical layer adjoining it; large cavity at *x* being filled with hair-like callus cells from bordering cells. Cavity due to excessive enlargement of cortical cells with resultant tearing of middle lamellae and plasmodesmata. Other evidences of internal callus.

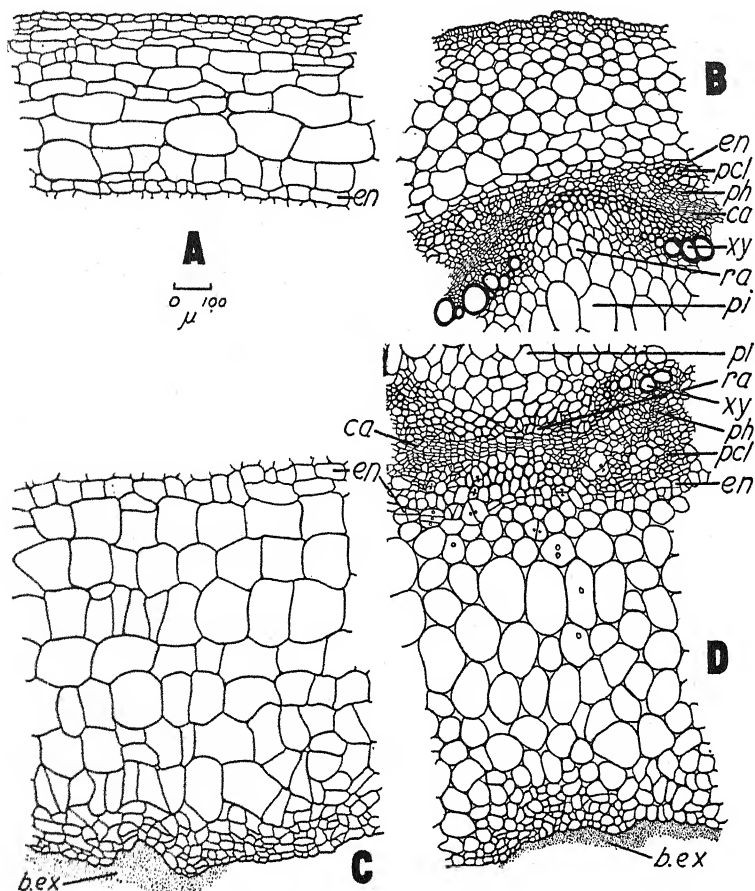


FIG. 22.—Sections through hypocotyl treated unilaterally with bacterial extract (*b.ex*). Same age as other sections of figs. 21 and 22, 8 days after treatment. *C, D*, longitudinal and transverse sections of treated side; *A, B*, exactly opposite, not treated. Note general diameter increase in *D*, enlargement of cortical cells, especially in radial and tangential planes; increase in intercellular space in cortex; less enlargement but more division immediately below *b.ex*; cell division in endodermis (*en*), pericycle (*pcl*), cambium (*ca*), phloem (*ph*), ray (*ra*), and pith (*pi*); less differentiation and maturation in *B* and *D* than in *B* of fig. 21; change in orientation of cell axis comparing fig. 21*A* with 22*A, C*, and fig. 21*B, C* with 22*B, D*.

capitated and heteroauxin treated second internodes of the Red Kidney bean by KRAUS, BROWN, and HAMNER (16). When lateral roots developed in or near the treated region, cortical cells abutting the endodermis, the endodermis, pericycle, and rays seemed to be involved in their initiation as reported for secondary roots of the Leguminosae and Cucurbitaceae by JANCZEWSKI (10).

The small cells of the epidermis and the adjoining cells evident in figure 22A-D indicate that cell division has taken place in the outer cortex. Whether these and other cell divisions noted in treated hypocotyls are direct responses to the applied heteroauxin or are secondary reactions to initial cell enlargement and other effects induced by the bacterial extracts and by heteroauxin has not yet been determined. There are many cells, especially in the endodermis and adjoining cortical layer, which enlarge and then go through a series of divisions without continued enlargement. Further study of the material fixed hourly immediately after treatment may reveal that cell division is an initial response to certain concentrations of these substances. WEHNELT (33) and JOST (11) assert that it is.

The affected tissues are less differentiated than the normal. In the specimen represented in figure 22A-D differentiation is less advanced than in the untreated specimen shown in figure 21A, B. The endodermis has dedifferentiated; and differentiation of the stele is below normal. Phloem fibers have failed to mature, as have many of the prospective woody constituents of the xylem.

The sections of figures 21C and 22D reveal that the effects were most pronounced below the site of application. This probably is due to greater concentration of the substance (*b.ex*) in that region. It probably indicates that other effects may be anticipated from different dosages and concentrations. Immediately below the site of application of 3 per cent paste and of the bacterial extracts, there was less swelling than at the periphery of the application. There also was more evidence of division in the outer cortex (figs. 21C, *b.ex.*; 22C, D). This indicates that high concentrations tend to induce less cell enlargement (or none) and more (or only) cell division. Since high concentrations seem to induce more cell divisions than low ones, the high incidence of divisions in the stele, endodermis, and adjoining row of cortical cells may indicate an increased concentration in this

region, possibly owing to meeting of the autoauxones and the heteroauxones at the endodermal barrier.

### Discussion

The results indicate that in the Red Kidney bean the cotyledons and young epicotyl are initial sources of growth hormones; further, that all aerial vegetative parts if green are able either to generate growth substances or to convert their precursors into hormones. This inference is based on the observed increase in length of decapitated hypocotyls in the light and of plants both decotyledonized and deepicotyledonized. Transport of growth substances proceeds mainly toward the central axis from the peripheries of the aerial divergencies. In the central axis transport is mainly basipetal but also acropetal and transverse. While decapitated hypocotyls and first internodes elongate, decapitated second and third internodes do not. Possibly those here employed were too old, even though some were only 1 cm. long when decapitated. Most were 1.5 cm. long at that time.

The inward and downward flow of hormones or hormone precursors from divergences into the axis is somehow associated with inhibition of development of axillary buds of the cotyledonary and higher nodes. This effect is demonstrated by development of these buds after spontaneous blasting of the leaves of the embryonic plumule or after decapitation of the internodes. Inhibitory effects of the intact leaf are evident in increased elongation of first internodes after petiole decapitation.

The observation that leaves fold upward whether treated on the upper or lower side needs further investigation, also the observation that application of heteroauxin to the upper side of a leaf leads to lengthening of the petiole while application to the lower side leads to shortening. Possibly position of xylem and phloem in the veins and petioles is in some way associated with the phenomenon.

The bean is highly disposed following wounding to formation of surface and internal callus and their derivatives; to adventitious root development and root fasciation; and to root regeneration. It is not disposed to formation of adventitious shoots or to non-nodal shoot regeneration. Moderately old hypocotyls grown above soil

and later encased in sand will develop roots in four rows throughout their full length if the sand is kept moist. Internodes failed to respond to this treatment with root development.

No detailed discussion will be attempted of implications of these results relative to polarity and correlation in the bean; that will be done in a later paper. The present report is concerned mainly with the etiological implications of the experiments and data. These exemplify basic principles discussed earlier (21, 22). In the second paper, hormones acting as external agents were recognized as pathogenic factors. The experiments and data of the present paper show clearly that disturbances in plants following application of a foreign chemical agent, heteroauxin, with or without wounding, are due to a causal or etic complex, which comprises (a) the foreign agent, and (b) the disposition of various cells, tissues, tissue systems, and organs of the plant to react at a particular moment to a particular concentration and amount of the agent. Four factors that condition the disposition of a cell, tissue, tissue system, or organ to react may be recognized: (1) hereditary constitution, genic and non-genic; (2) past experience; (3) correlative factors (effect of one part on another part); (4) environal factors (factors outside the whole affecting it at the moment under consideration). The relation of these factors to one another is schematically treated in an earlier paper (21).

Individuals of different species, bean or tomato, reacting differently to the same concentration or amount of heteroauxin, indicate the role of hereditary factors, genic and non-genic.

Differential reaction of the same organ, tissue, or cell of the bean at different stages of its development or following different culture conditions (light, humidity, temperature) prior to treatment with a given concentration of heteroauxin indicates the role of the past history factor.

The role of correlative factors is indicated by differential susceptibility of the same organ, tissue, or cell of the bean to a given concentration of heteroauxin in the presence or absence of other members of the whole.

Differential reaction of the same organ, tissue, or cell of the bean to the same concentration of heteroauxin under different environ-



al conditions at and subsequent to the moment of application indicates the role of environal factors.

Given (a) disposition to react to a particular inciting agent, in this case an external one, in the form of a particular concentration of heteroauxin, and (b) the application of this concentration, then (c) a characteristic reaction, cell enlargement with or without division, or possibly cell division alone, sets in. Next this event, playing the role of an internal factor, may become the inciting factor which releases other reactions according to the dispositions of the cells involved. This causal complex gives certain tissues and cells opportunities to realize qualitative and quantitative potentialities of cell enlargement, division, and differentiation which are not stimulated to or are inhibited from expression in the course of normal development.

Usually the inciting agent or factor, especially if it is an external one, is called the cause (22). Brevity is the only justification of this usage. It ignores the fact that each constituent of the etic complex is a necessary but not a sufficient cause of the event. Each in turn will appear as the cause if added or withheld while the others are kept constant.

These considerations emphasize the role of living protoplasm in reactions induced by heteroauxin and other growth substances, a consideration repeatedly stressed by JOST (11-13). However, they do not commit one in the stimulus dispute carried on by FITTING,<sup>4</sup> WENT (34), and JOST.

Since wounding or application of bacterial extracts which contain heteroauxin (and possibly other growth substances) and application of pure  $\beta$ -indoleacetic acid lead to similar effects in the bean and tomato, the hypothesis is suggested that disturbance of the usual growth hormone concentration of the affected plant is one constituent of the causal complex of the cell enlargement and division characterizing these effects. Their induction by application of heteroauxin and the apparent partial substitutability of heteroauxin for the auxines of the cotyledons and epicotyl suggest that the local growth substance concentration in the region of the prospec-

<sup>4</sup> FITTING, H., Die Hormone als physiologische Reizstoffe. Biol. Zbl. 56:69-86. 1936.

tive tumor rises above the norm, but that this hyperauxony is not sufficient either to inhibit cell enlargement or division or to induce necrosis. This part of the hypothesis could readily be tested by comparing the auxone contents<sup>5</sup> of tumors, galls, and normal tissues.

Disposition of the affected organ, tissue, cell, or cell part to respond with enlargement, division, or differentiation to a particular hyperauxony is the other constituent of the causal complex of growth reaction to wounding, bacterial extracts, and heteroauxin applications.

In gall formation the same etic complex is operative but in addition a similar causal complex for the infecting agent must be considered (21, 22). If the organism consists of only one cell, intercellular correlative factors do not enter the complex so far as the parasite is concerned. In gall formation, the hyperauxonism probably is not a brief but an extended condition due to continued presence of the parasite and the disturbances which it induces. The parasite probably not only furnishes a more or less continuous supply of heteroauxones, but through abnormal growth or lethal effects of the host disturbs normal production, activation, and transport of autoauxones. In starting new and abnormal growing centers, the parasite probably creates additional sites of increased autoauxone

<sup>5</sup> The field of growth substances is in need of a tentative classification and nomenclature. Since the terms auxin and heteroauxin have been applied to specific chemical agents, and since diverse chemical agents act as growth substances, the collective term auxins is not satisfactory. Proceeding from the Greek verb *auxein*, which means any increase, amplification or waxing, we have used auxone as a generalized term for all growth substances, including the reproductive hormones that affect growth. It includes the A and B growth substances. In case specific auxones should be found that induce dimensional increase (cytoauxesis), numerical increase (cytomeresis), complexity increase (cytoplasesis), or plasma increase (cytoplasmesis) of cells, they can be designated cytoauxones, etc. Any auxone inducing more than one of these reactions may be said to be cytoauxogenic, cytomeregenic, etc. Auxones influencing reproduction may be designated genesones. Excess or deficiency in any of these processes or substances may be indicated by the prefixes hyper or hypo respectively.

Some auxones are produced by the individual whose development they affect; these are autoauxones (auxin a and b). Others apparently do not affect growth of the plant producing them but of other plants; these are heteroauxones (heteroauxin). Heteroauxones also include growth substances not known to occur in plants (indolepropionic acid, indolebutyric acid, etc.).

No confusion will arise if the ending 'one' is avoided in coining specific names of growth regulating substances.

production. Internal wounds resulting from death of some gall cells may induce local hyperauxonity, with resultant cell enlargement and division, as in simple wounding. Since *P. tumefaciens* usually becomes initially effective only through wounds, its presence may in part enhance and extend spatially and temporally a hyperautoauxony instigated by the initial wound.

In the highly differentiated galls incited by some insects, the kind of auxones produced by, in, or on the parasite as well as the mode, duration, and quantity of their production probably are more significant than in the relatively simple callus-like galls (18) induced by *P. tumefaciens*. It is possible that the normal bacterial flora of the exterior and of the intestinal tract and mouth parts of insects play roles in the production of heteroauxones on or within the insect's body, or that the insect itself produces auxones in the course of its metabolism. If heteroauxones are produced from ingested food material within the insect's intestinal tract, the frass of insects should contain heteroauxones. LARUE's report (19) that feces of insects produce tumors suggests that this hypothesis may be a step in the right direction.

Since heteroauxones produced by non-gall forming organisms incite tumor formation in plants, not parasitized by these organisms, and since a synthetic heteroauxone or wounding may lead to tumor production, it appears that gall production in a particular host by a particular organism is initially conditioned by factors determining specificity of parasitism or infection rather than by specificity of a chemical mechanism involved in growth disturbances.

The event of infection must be subjected to the analysis here outlined. Its causal analysis involves consideration of two interacting causal complexes, the host, organ, tissue, cell, or cell part being infected and the individual, organ, tissue, or cell doing the infecting. For the first, the disposition to be or not to be infected by a particular species, individual, organ, tissue, or cell must be analyzed in terms of the four causal constituents just discussed. For the infecting agent, its disposition to infect or not to infect the particular individual or part under consideration must be resolved in terms of these four factors (21, 22).

Obviously capacity to infect and capacity to incite growth re-



sponses or other pathic effects are not due to identical causal complexes, nor are capacity to be infected and capacity to respond to infection with one or more of the various manifestations of injury or injury reaction.

Many observations indicate that infectivity must be differentiated from more severe types of pathogenicity or virulence (22). The least severe obvious injury resulting from infectivity is loss of purity or contamination of the host and of the parasite. COOK (6) has shown that tomato plants may be heavily infected with *Fusarium lycopersici* without displaying other obvious evidences of injury. If this fungus does not produce its characteristic toxic substances, the symptoms characteristic of tomato blight do not develop. KANE and LINK (14) showed that while *F. lycopersici* is host specific as to infectivity, its toxic substances are not in that they induce typical blight and wilt injury in plants never parasitized by the fungus.

The observation that fungi and bacteria that incite galls produce heteroauxin suggests the hypothesis that heteroauxin or other heteroauxones play important roles in the production of galls by parasites. Studies are under way to determine whether *Erwinia amylovora*, *Phylomonas rhizogenes*, *Rhizobium phaseoli*, *Taphrina deformans*, other species of *Taphrina*, and *Ustilago zeae* produce heteroauxin or other heteroauxones in culture.<sup>6</sup> Possibly mycorrhizal fungi serve their host by the formation of heteroauxones.

Studies also are under way to determine whether galls such as the clubs induced by *Plasmodiophora brassicae*, the nodules of the bean, leaves and fruits infected with *Taphrina* spp., rust, smut, and virus

<sup>6</sup> Note while reading proof:

Using the procedure and apparatus described by GALLAGHER, KOCH, and DOREMAN (GALLAGHER, T. F., KOCH, F. C., and DOREMAN, R. I., Procedure for quantitative extraction of sex hormones from urine. Proc. Soc. Exp. Biol. and Med. 33:440-444. 1935), ether extracts were prepared from these organisms. Preliminary tests gave the color reaction (ferric chloride, hydrochloric acid, amyl alcohol) characteristic of  $\beta$ -indoleacetic acid for these organisms in the following ascending order: (1) *E. amylovora*, (2) *P. tumefaciens*, (3) *Ustilago zeae*, (4) *Rh. phaseoli* (strain 400 Wis.), (5) *Taphrina deformans*, (6) *P. rhizogenes*, (7) *Rh. phaseoli* (strain 407 Wis.), (8) *Rh. phaseoli* (strain 403 Wis.). We have not had time to make the coleoptile test. We are indebted to H. R. ROSEN for culture of no. 1; E. C. STAKMAN for culture of no. 3; E. B. FRED for cultures of nos. 4, 7, 8; A. J. MIX for culture of no. 5; and to A. J. RIKER for culture of no. 6. We are indebted also to PROFESSOR KOCH for use of his laboratory and equipment and for advice and help.

infected organs, and several insect galls have a higher auxone content than the healthy organs.

The observation that high concentrations of auxones bring on necrobiosis and necrosis suggests that fungi and bacteria may necrotize by contributing to lethal hyperauxony, through adding their heteroauxones to the host's autoauxones, stimulating host production of autoauxones, or blocking autoauxone transport and causing local accumulation.

That heteroauxin applied to a plant can either substitute in part for its autoauxones or augment their action, and the well known fact that soil fungi and bacteria produce heteroauxin, suggest that some of the beneficial effects of humus soil may be due to the auxones of decaying plant debris or soil flora.

Auxones are harmful to roots in relatively high dilutions, and roots of the bean treated with certain concentrations of heteroauxin resemble those of certain bog and muck soil plants. This suggests that hyperauxony of these substrates may possibly be in part responsible for the characteristic roots of some of the plants grown in them.

Given a foreign organism able to infect a particular host and capacity of this parasite to form heteroauxin, the presence of the parasite need not lead to gall production if the metabolic status of the infected tissue is such that relatively little tryptophane or tryptophane containing proteins is present. The metabolic status and nutrition of both the parasite and host and everything that influences them undoubtedly are factors in the etic complexes of galls.

It has long been surmised that gall incitants may be effective by means of chemical agents. MALPIGHI in 1672 (26) devoted a portion of his anatomy of plants to galls. He attempted an explanation along physiological and chemical lines. He postulated that juices introduced by the insect act as foreign chemical agents which lead to a new fermentation (in modern terms chemical change) or to a new movement (in modern terms electronic vibrations, etc.) in the injured region. Nutritive sap accumulates abnormally in this region. The local disturbance in turn incites fermentation in this accumulated sap leading to the dimensional and numerical increase in parenchymatous cells which he had observed in the galls. Here we

have a physiological chemical theory by the first of the pathological plant anatomists and phytoparasitologists. MALPIGHI also concerned himself with legume nodules but was unable to discover any insect within them, although he reported a peculiar content.

The mechanism whereby *P. tumefaciens* induces galls destructive in economic plants has stimulated much speculation and much research. SMITH was engrossed in these endeavors and did much to indicate that the organism might be effective through chemical agents. His last book (31) contains an excellent summary and full bibliography. BECHHOLD and SMITH (2) reported finding a "tumefaciens plastin." NĚMEC (27) reported that bacteria produce callus forming substances. This phase of the crown gall problem is reviewed by RIKER and BERGE (28) and needs no further review here.

HABERLANDT's findings (8) of wound hormones and recent hormone work indicate definitely that hyperauxony may be a factor in tumor and gall production. Demonstration that many fungi and bacteria produce  $\beta$ -indoleacetic acid in the course of their metabolism is highly suggestive that this and similar substances may be some of the chemical agents effective in gall formation.

In our work so far, the Salkowski color test, which is considered specific for  $\beta$ -indoleacetic acid (1, 15), and the comparable effect on plants of heteroauxin and the bacterial extracts are the basis for assertion that *P. tumefaciens* produces heteroauxin. Chemical identification by purification and analysis are essential for final proof. Further work is necessary to determine whether heteroauxin is the only auxone formed by *P. tumefaciens*. Possibly the better extraction technique now employed will enable us to obtain greater yields. They must be larger to permit analysis, because KÖGL and KOSTERMANS (15) started with 50 kg. of yeast and finished with only 9 mg. of  $\beta$ -indoleacetic acid.

Our gross observations on the bean confirm JOST and REISZ's (12) more refined findings for *Avena* coleoptiles, that both concentration and amount of auxone are factors in the response; that heteroauxone enters the plant within the first two hours, and that it moves upward, downward, and transversely; that heteroauxones may substitute for or augment a plant auxone; and that temperature and moisture are conditioning factors. They also show that the heteroauxin effect is greatest in the dark.

Heteroauxin apparently does not pass heavily cuticularized or suberized walls. Possibly this accounts for the indicated accumulation of auxones in the endodermal region of plants to which heteroauxones are applied.

The histologic studies of the hypocotyls indicate that the lower concentrations of auxones induce auxesis, while higher concentrations induce auxesis and meresis. The higher concentrations also are able to modify plasesis leading to dedifferentiation and redifferentiation (metaplasesis) of the endodermis, probably by influencing cell enlargement and division. Through these effects they also lead to hypoplasesis of the stele, evident in less development of secondary xylem and phloem and diminished differentiation and maturation of primary xylem and phloem constituents. Secondary hyperplasia is evident in the extrafascicular bundles observed by us for the hypocotyl and reported for the internodes by KRAUS and associates (9, 16).

Heteroauxin not only induces functional injuries but indirectly through growth disturbances leads to material injury in the form of tearing of middle lamellae and plasmodesmata in the formation of cortical cavities. The mechanism of necrosis of leaf tissues has not yet been investigated. Whitening and yellowing indicate that chlorophyll is either destroyed or its synthesis stopped by certain concentrations of heteroauxin. The effects of this one substance in different concentrations and amounts on the various organs of the bean illustrate practically all the major types of functional and material injuries<sup>7</sup> known to the phytopathologist.

Are not the results of these experiments further indications that circumscription of phytopathology on the basis of the germ theory of disease has outlived its usefulness and that mycologic concepts and techniques alone are not adequate for its development? (21, 22).

### Summary

1. Differential behaviors of axes of bean, tomato, and *Bryophyllum* inoculated with *Phytophthora tumefaciens* are due probably to differences in growth patterns of the axes of these plants.

<sup>7</sup>Injury designates destruction or other impairment of functional capacity (BOT. GAZ. 97:686-688, 1936).

2. Much of the overgrowth may be due to responses to wounds essential to the course and completion of the infection process.

3. The bean is highly disposed following wounding to formation of surface and internal callus and their derivatives, adventitious roots, and root regeneration. The roots are disposed to fasciation. The bean is not disposed to adventitious shoot formation or to non-nodal shoot regeneration. Moderately old hypocotyls developed in air and then encased in wet sand will develop adventitious roots through the full length of the organ.

4. Occurrence of these events at the wound and above it indicates that growth substances play a role in their enactment.

5. Growth substances apparently are formed or activated in all aerial vegetative members of the bean and transported to the central axis where they move mainly downward, but upward and transversely as well.

6. Elongation of hypocotyl, internodes, and development of axillary buds are retarded or inhibited by hormones from divergences of segments of the axis above them.

7. Disposition of an organ to enact these events is conditioned by its hereditary constitution, age, presence or absence of other organs, metabolic status, and environal factors (temperature, moisture, and light).

8. Enactment of these events is influenced by experimentally introduced growth substances (heteroauxones). These seem to be able to augment or to substitute in part for the native growth hormones (autoauxones) of the bean.

9. Different concentrations and amounts of heteroauxin applied to different organs of the bean and tomato under different conditions are able to elicit almost the entire gamut of injuries and injury reactions known to the phytopathologist. This includes tumors.

10. *Phytophthora tumefaciens* produces  $\beta$ -indoleacetic acid (heteroauxin) when grown in dextrose-tryptophane or in dextrose-tryptophane peptone liquid or solid (agar) medium. The crude ether extract of the non-inoculated media does not contain heteroauxin. The chemical Salkowski and the biological *Avena* coleoptile tests were used.



11. The crude ether extract elicits symptoms from bean and tomato similar to those induced by inoculation with *P. tumefaciens* or by treatment with heteroauxin.

12. Heteroauxin is a chemical agent by means of which, possibly in conjunction with others, *P. tumefaciens* incites galls.

13. Unequal amounts of 3 per cent heteroauxin paste and of crude extract of *P. tumefaciens* applied to the hypocotyl of the bean elicit practically identical histic and cytic effects. These are cell enlargement, cell division (possibly a consequence of enlargement), and as a consequence of these suppression of normal differentiation and maturation but genesis of new meristems with new differentiations in abnormal sites. Schizogenous cavities are formed, followed by filling with peripheral callus. Differential response by the same tissue or cell to different amounts and concentrations and different responses of different tissues or cells to the same concentration or amount are indicated. Most of the abnormal tissues and cells are pathic in the sense that they have less adaptive capacity for unfavorable environal conditions than have the normal healthy ones.

14. This study indicates that biology must use the concept "causal complex" in attempting analysis of problems in normal and abnormal, healthy and pathic morpho-, organo-, histo-, and cyto-genesis. Each constituent of the causal complex is a necessary but not a sufficient cause for the event under consideration.

15. A tentative nomenclature and classification of growth substances are proposed.

16. Hypotheses are advanced suggesting partial explanation of legume nodules, insect galls, forms of roots of bog, muck and mycorrhizal plants, as well as beneficial effects of humus soils, in terms of auxones.

17. Relation of infectivity and other expressions of pathogenicity are discussed.

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## CURRENT LITERATURE

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*Methods in Plant Physiology, a Laboratory Manual and Research Handbook.* By W. E. LOOMIS and C. A. SHULL. New York: McGraw Hill, 1937. Pp. xviii+472. Figs. 92. \$4.50.

As stated in the preface, this book is an attempt to bring together material which will be useful to the teacher and student in plant physiology, as well as to the research worker in this and allied fields. To a large extent these purposes are fulfilled.

The first 227 pages are devoted to a description of 182 standard experiments suitable for a laboratory course in plant physiology, including experiments relative to water relations of plants, plant nutrients, diffusion, colloidal phenomena, photosynthesis, pigments, foods, respiration, enzymes, growth, and movement. Following the introduction to each chapter, a limited number of pertinent references are cited. A few references relative to certain experiments follow the description directly. Each experiment is followed by one to four thought-provoking questions.

Part II of the text contains directions for biochemical, biophysical, and biometrical determinations which are incident to research in plant physiology. These descriptions give rather detailed information in regard to equipment, cleaning of glassware, filtration, titration, standardization of solutions, chemical analysis of plant materials, freezing point and H ion determinations, and control of light, temperature, and moisture. The last chapter contains thirty-four pages of description and discussion of statistical methods by G. W. SNEDCOR. A copious appendix of forty pages completes the text.

The language used in the directions and explanations is clear and concise, a commendable factor too frequently lacking in such manuals and textbooks.

In the advanced portion of the text the general procedure which is essential to the problem in hand is carefully outlined, while the general details are left to the discretion of the investigator. This is as it should be, for although certain of the details of any line of investigation may change from time to time, the basic procedure remains the same and its exact fulfilment is essential for significant results in research.

The chapter on statistical methods is a valuable addition in supplement to the text. The student of plant physiology should have at least a brief introduction to this subject, since at the present time statistics apparently occupy an important place in the interpretation of research in biology.

The appendix is one of the most useful parts of the book. In it considerable information and data essential to the study of plant physiology are assembled and condensed into tables.

It is impossible to include in any text all the material that every worker in the field would desire, or to express the knowledge therein to the satisfaction of all. In some cases, however, it seems to the reviewer that the experiments are outlined in a more complex fashion than is necessary. This is illustrated in the directions for the preparation of invertase from yeast. For an intermediate experiment this could be done just as efficiently and in a much less elaborate manner than as described in the text.

The directions for the experiments assume that the average laboratory of plant physiology has all the equipment designated. It is perhaps best, however, to give directions calling for ideal equipment, and then leave to the ingenuity of the instructor the task of replacing or substituting when necessary.

The objections that may be pointed out in the text are minor, however, and the book in its entirety is an excellent one. It is a volume that all who are interested in plant physiology or its allied fields will wish to possess.—E. C. MILLER.

*Lehrbuch der Pharmakognosie für Hochschulen, fünfte vollständig umgearbeitet und vervollständigte Auflage.* By GEORGE KARSTEN and ULRICH WEBER. Jena: Gustav Fischer. 1937. Pp. vii+420. Figs. 574.

This standard work, now in its fifth edition, has been the principal textbook of pharmacy in Germany for more than twenty years. Even a cursory glance through the book, with its numerous illustrations, many of which are colored, shows the advanced condition of the subject in German universities. KARSTEN, now Professor Emeritus of Botany of the University of Halle, made notable morphological contributions before he began to specialize in pharmacy. His work on the life histories of seed plants and of diatoms is classic, and his extensive work on marine plankton has not yet been surpassed. This broad training has been utilized in the textbook, making more definite and reliable the diagnosis of constituents of drugs. WEBER is giving courses in pharmacy in the University of Würzburg.

The general arrangement of drug plants and the drugs obtained from them follows the taxonomic sequence from the algae to the orchids. There is usually a general description of the plant, followed by morphological and histological characters, and often there is added the microscopic structure of the drug. For the angiosperms, which furnish so much of the material for drugs that all other sources form a comparatively small percentage, there is a subdivision into drugs derived from the root or rhizome, from the stem, bark, leaf, flower, fruit, seed, and other parts. Besides these detailed descriptions there are numerous keys for the determinations of various powders, those from root, stem, leaf, flower, fruit, seed, etc.; an arrangement based upon chemistry, a description of the comparatively few drugs obtained from animals, a list of homeopathic drugs, and an arrangement based upon the diseases for which the drugs are used.

The clear presentation, with such a wealth of illustration of plants in the normal condition and as they appear in drugs, together with complete descrip-

tions of solutions, make the book a good introduction to scientific pharmacy.—  
C. J. CHAMBERLAIN.

*Genetical and Taxonomic Investigations in the Genus Oenothera.* By R. RUGGLES GATES. London: Phil. Trans. Roy. Soc. Series B. Vol. 226 (no. 536). 1936. Pp. 239-355. Figs. 73.

This is a presentation of the beginning of a survey of the genus *Oenothera*, mainly in eastern Canada and adjacent territory, through a combination of genetic and taxonomic methods. Seventeen new species, fifteen new varieties, and many smaller variations are described as a result of three years of pedigree cultures from about 100 collections of wild seeds from different localities. In addition, seven previously described species have been recognized in new localities.

New information upon the geographic distribution and relationships of the various forms is presented, indicating a south to north movement in several different lines, and also a coastal series of forms. Six species have produced known or probably trisomic mutations, one species a triploid mutation, and one a periclinal chimera, most of these mutations being directly from wild seeds. Some of the species show "early" and "late" development in the cultures, but the condition is not inherited and appears to be an epharmonic response to environmental conditions occurring at an early stage of the young plant's development. And in several species a new category of evanescent characters appears.

Cytological examination has shown that all these forms possess, without exception, a ring of fourteen chromosomes.

The polymorphic character of the genus, even in areas such as Nova Scotia which were heavily glaciated and apparently contain no other endemic species, leads GATES to conclude that evolution has proceeded more rapidly in this genus than in most others. Gene mutations have been active in supplying the materials for specific differentiation and crossing has also played a part in increasing the number of specific types, since owing to catenation the hybrids breed true. Parallel mutations have also occurred frequently in the different species.—J. M. BEAL.

*The Nature and Properties of Soils.* By T. L. LYON and H. O. BUCKMAN. 3rd ed. New York: Macmillan Co., 1937. Pp. xiii+392. Figs. 45. \$3.50.

Investigators and teachers of plant and soil science will welcome this new edition of a standard and important text, the first edition of which under its present authorship appeared in 1922. The treatment in the present edition follows the general plan of the preceding ones. The nature and properties of soils are not merely treated as such but they are also related to the growth of plants. Edaphology is the name applied to this phase of soil science, in contrast to pedology, which treats soil science as a pure science and does not make the application to the plant. The present edition brings the subject up to date. Considerable

new material has been added. One whole chapter is devoted to the newer knowledge of the nature of colloidal clay and of base exchange. Considerably less space is given to soil water than in the second edition.

It is interesting to note the development of edaphology as reflected in the relative emphasis of the three editions on various subjects. For example, base exchange received relatively little attention in the first edition, while a chapter each was devoted to soil aeration and soil temperature. The latter two subjects are given rather slight consideration in the two later editions, and are treated in connection with other subjects. On the other hand, the important and rapidly developing subject of base exchange is given much emphasis, especially in the last edition. While it is essential that the newer developments be given adequate treatment, it seems unfortunate that in so doing the consideration of other important subjects should be so severely curtailed.—S. V. EATON.

*Die kontraktile Zelle der Pflanzen.* By SILVIA COLLA. Berlin: Gebrüder Borntraeger, 1937. Pp. ix+168. Figs. 77. Rm. 12.

This work is volume X of the Protoplasma Monographien. The introduction develops the historical and current points of view regarding contractility. The first chapter discusses the material and the research methods used in the study of cellular contractility. Other chapters discuss the morphology of contractile cells, the mechanisms of movement of contractile organs, the various phases of contraction, the laws which govern the contraction and movement of isolated cells, the physico-chemical phenomena during contraction, and a comparison of plant cell contractility with other varieties of contraction, such as striped muscle contraction, contractile protozoa, etc.

Chapters V and VI are especially interesting. The former deals with various kinds of stimuli—electrical, mechanical, thermal, radiant, osmotic, and chemical. It also takes up the problems of intensity of stimulation as threshold phenomena, "all or none" relations, summation, tetany, fatigue, refractive stages, etc. The latter considers such changes in the protoplasm as variations of potential, pH and rH, and modifications in the concentration of crystalloids, colloidal state, and protoplasmic permeability as concomitants of contraction.

The book gives a well rounded consideration of its field, and is written in engaging style. A long list of citations offers opportunity for wide reading by those interested in research on such problems.—C. A. SHULL.

*Die chromatographische Adsorptionsmethode.* By L. ZECHMEISTER and L. V. CHOLNOKY. Vienna: Julius Springer, 1937. Pp. ix+231. Figs. 45. Rm. 14.4.

About 30 years ago TSWETT used the chromatographic method for separating mixtures of carotenoids. The method is now widely used, and this monograph, which gives a detailed account of it, should stimulate even wider application of the technique to research in the fields of plant and animal chemistry.

The monograph is divided into two parts, general and special. There are two chapters devoted to general considerations, such as the fields in which the chromatographic method is useful, the history of its use, the fundamental theoretical basis of chromatography, the relation between molecular constitution and chromatogram formation, and the details of the method as used with both colored and colorless compounds. The special part, presented in three chapters, takes up the application of the method to naturally occurring pigments, such as chlorophyll, porphyrins, bile pigments, carotenoids, naphthaquinones and anthraquinones, flavines, pterine, anthocyanins, and other unclassified pigments; to artificial pigments and dyes; and to colorless and weakly colored substances. Among the latter are found various aliphatic and aromatic compounds, benzol derivatives, polycyclic compounds, certain plant and animal poisons, alkaloids, enzymes, vitamins, hormones, tannins, and pharmaceutically useful drugs.

Several hundred citations and author and subject indexes make the work extremely useful and usable. It will commend itself particularly to students of the polyene carotenoids, and hormones.—C. A. SHULL.

*Oklahoma Flora.* By THOMAS R. STEMEN and W. STANLEY MYERS. Oklahoma City: Harlow Publishing Corporation, 1937. Pp. xxix+706. Figs. 494. \$6.00.

Amateur botanists and beginning students of biology and botany in Oklahoma should be grateful to the two teachers in the Oklahoma City school system who have expanded their earlier work, *The Spring Flora of Oklahoma*, into the present volume. It seems to be intended primarily for the use of students. Over 1600 species are keyed and described, of which almost 500 are illustrated with original and borrowed line drawings. Tables listing edible, poisonous, and drug plants, those that should be protected, those causing hay fever, and those useful in fish culture and for transplanting are included, to appeal to the general public.

Professional botanists will regret that, in a work of such a pretentious nature, no attempt has been made to treat any grasses, sedges, or rushes, even though other species with only one known station in the state are included. Unfortunately the authors have been inconsistent in this latter respect, so that even though the work is said to be complete, numerous species have been omitted for which herbarium specimens are easily available. It is to be hoped that in any revision the authors will use all important available collections as a basis.—C. E. OLMSTED.

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